



# Investigation of the Inheritance of Polycomb Group-Dependent Repression through Mitosis

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## **Investigation of the inheritance of Polycomb Group-dependent repression through mitosis**

### **Abstract**

Inheritance of gene expression patterns through multiple rounds of cell division is crucial for the normal development of multi-cellular organisms and is mediated by epigenetic mechanisms. Many epigenetic mechanisms are believed to involve heritable changes to chromatin structure. This includes maintenance of transcriptional repression by Polycomb Group (PcG) proteins. It is unknown how PcG-dependent repression is maintained during or re-established after mitosis, a process that involves many physical and biochemical changes to chromatin. Understanding the behavior of PcG proteins during mitosis is key to answering this question: if PcG proteins remain bound in mitosis they may constitute the memory themselves, or else transcriptional memory must reside elsewhere, such as in the altered chromatin structures induced by PcG proteins.

PcG protein association with chromosomes in mitosis in *Drosophila* S2 cells was examined by immunofluorescence and cellular fractionation. PcG proteins are

associated with mitotic chromosomes, which is consistent with a role in carrying information about transcriptional repression through mitosis.

Localization of PcG proteins to specific sites in the genome was assessed by chromatin immunoprecipitation (ChIP) followed by genome-wide sequencing (ChIP-SEQ) on mitotic cells. A method for isolating pure populations of mitotic cells was developed to assess PcG protein localization in mitosis unambiguously. PcG proteins were not detected at well-characterized PcG targets including *Hox* genes on mitotic chromosomes, but a covalent modification of histone H3 associated with PcG-dependent repression, trimethylation of lysine 27 (H3K27me3), is retained at these sites. Two PcG proteins Posterior Sex Combs (PSC) and Polyhomeotic (PH) remain at about 10% of their interphase binding sites in mitosis. PSC and PH are preferentially retained in mitosis at sites that overlap recently described borders of chromatin domains (1), including sites that overlap domain borders flanking *Hox* gene clusters. These persistent binding sites may serve to nucleate re-establishment of PcG binding at target genes upon mitotic exit, perhaps with assistance of H3K27me3.

Thus PcG proteins may form part of the transcriptional memory carried through mitosis, but perhaps not by persistent association at the targets of repression. Retention of elements at chromatin boundaries in mitosis may serve as a general mechanism for epigenetic memory.

1. Sexton T, *et al.* (2012) Three-dimensional folding and functional organization principles of the *Drosophila* genome. *Cell* 148(3):458-472.



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**replication in vitro**

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# **Chapter 1**

## **Introduction**

## Epigenetics

Cellular differentiation in multi-cellular organisms involves changes in gene expression that give rise to numerous cell types with distinct identities. While the genome within each cell of an organism is identical, the gene expression profile of a cell type is unique. Thus a cell's identity may be considered to reside in its gene expression profile. Differentiation, the process by which cell identities are acquired, is a central part of normal development. After differentiation, cellular phenotypes, and the gene expression patterns that underlie them, must be maintained through cell divisions, often for the lifetime of the organism (Probst et al., 2009).

Maintenance of stable gene expression patterns during and after differentiation occurs through epigenetic mechanisms. Epigenetics can be defined as “the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence (Riggs et al., 1996).” Often the absence of the signal or event that initiated the change is used as an indication of an epigenetic change (Ptashne and Gann, 2002). Many epigenetic mechanisms are believed to involve heritable changes to chromatin structure. Changes to chromatin structure include higher-order chromatin folding, post-translational modification of histone proteins, incorporation of histone variants, nucleosome positioning and binding of non-histone chromatin proteins (reviewed in Ng and Gurdon, 2008; Nightingale et al., 2006).

## The Polycomb Group

The proteins encoded by the Polycomb Group (PcG) genes are an example of epigenetic regulators that are thought to maintain heritable changes in gene expression through effects on chromatin.

### *Discovery*

The Polycomb Group genes were originally identified by their role in regulating the genes that control the development of body structures along the anterior-posterior (A-P) axis of the fruit fly *Drosophila melanogaster*. The body plan of the adult fly is subdivided into segments along the A-P axis, each of which has specific differentiated structures. Segment identities are determined by the gene products of the two clusters of homeotic (*Hox*) genes: the Bithorax Cluster (BX-C) and the Antennapedia Cluster (ANT-C) (Bender et al., 1983; Karch et al., 1985; Kaufman et al., 1980; Lewis, 1978). Specifically, the differential expression of the *Hox* genes along the A-P axis controls formation of different structures within each segment. Thus the precise boundaries of *Hox* gene expression must be established and maintained to ensure proper segmental development (reviewed in Maeda and Karch, 2009).

Mutations in the gene *Polycomb* (*Pc*) (Duncan and Lewis, 1982; Lewis, 1978; Lewis, 1947) the founding member and eponymous gene of the Polycomb Group, leads to ectopic expression of all three genes in the BX-C and two of the genes in the ANT-C (Beachy et al., 1985; Carroll et al., 1986; Celniker et al., 1989; Kuziora and

McGinnis, 1988; Riley et al., 1987; Wedeen et al., 1986), pointing to a role for *Pc* in repression of these genes in normal development. The ectopic expression of the *Hox* genes outside of their normal A-P boundaries leads to lethality during embryogenesis and homeotic transformations--transformation of one body part into another normally found elsewhere in the organism (Busturia and Morata, 1988; Duncan and Lewis, 1982; Struhl, 1981). The name of the gene, *Polycomb*, refers to the adult phenotype of heterozygous male flies. Segmental transformation of the second and third thoracic legs into the first thoracic leg causes the appearance of male sex combs, structures normally only found on the first thoracic leg, on the second and third thoracic legs (Duncan and Lewis, 1982).

Several other Polycomb Group genes were identified whose mutation causes homeotic transformation via misexpression of *Hox* genes in the BX-C and ANT-C outside of their normal region of expression. The *Drosophila* PcG genes are listed in Table 1-1. Many Polycomb Group proteins are conserved in most metazoans where they also function in *Hox* gene repression (reviewed in Schuettengruber et al., 2007).



**Table 1-1. Polycomb Group genes.**

Gene name	Symbol	Reference
<i>Enhancer of zeste</i>	<i>E(z)</i>	(Jones and Gelbart, 1990; Phillips and 1990; Wu et al., 1989)
<i>extra sex combs</i>	<i>esc</i>	(Struhl, 1981; Struhl, 1983)
<i>esc-like</i>	<i>escl</i>	(Wang et al., 2006)
<i>Su(z)12</i>	<i>Su(z)12</i>	(Birve et al., 2001)
<i>Polycomblike</i>	<i>Pcl</i>	(Duncan, 1982)
<i>Polycomb</i>	<i>Pc</i>	(Duncan and Lewis, 1982; Lewis, 1978; Lewis, 1947)
<i>polyhomeotic-proximal; polyhomeotic-distal</i>	<i>ph-p, ph-d</i>	(Dura et al., 1985; Dura et al., 1987)
<i>Posterior sex combs</i>	<i>Psc</i>	(Jürgens, 1985)
<i>Sex combs extra/ dRING1</i>	<i>Sce/dR</i>	(Breen and Duncan, 1986)
<i>Suppressor of zeste 2</i>	<i>Su(z)2</i>	(Adler et al., 1989; Wu et al., 1989)
<i>pleiohomeotic</i>	<i>pho</i>	(Gehring, 1970; Girtton and Jeon, 1994)
<i>pleiohomeotic like</i>	<i>phol</i>	(Brown et al., 2003)
<i>Scm-related gene containing four mbt domains</i>	<i>Sfmbt</i>	(Klymenko et al., 2006)
<i>Additional sex combs</i>	<i>Asx</i>	(Jürgens, 1985; Sinclair et al., 1998)
<i>calypso</i>	<i>calypso</i>	(Gaytan de Ayala Alonso et al., 2007)
<i>super sex combs</i>	<i>sxc</i>	(Ingham, 1984; Ingham, 1985)
<i>Sex comb on midleg</i>	<i>Scm</i>	(Jürgens, 1985)
<i>cramped</i>	<i>crm</i>	(Yamamoto et al., 1997)

While PcG proteins function to maintain the repressed state of *Hox* genes, another group of proteins is responsible for maintenance of active *Hox* gene expression, the trithorax group (trxG). Its founding member, *trx*, was identified in *Drosophila* by homeotic transformation that mimicked loss-of-function mutations in the *Hox* genes, suggesting *trx* was involved in activation of *Hox* genes (Ingham and Whittle, 1980). Other trxG proteins were identified by loss-of-function of *Hox* genes or in genetic screens for their ability to suppress PcG-dependent mutant phenotypes. This and subsequent analyses established trxG genes as antagonists of PcG genes (reviewed in Kennison, 1993; Schuettengruber et al., 2011).

#### *Evidence that PcG are epigenetic*

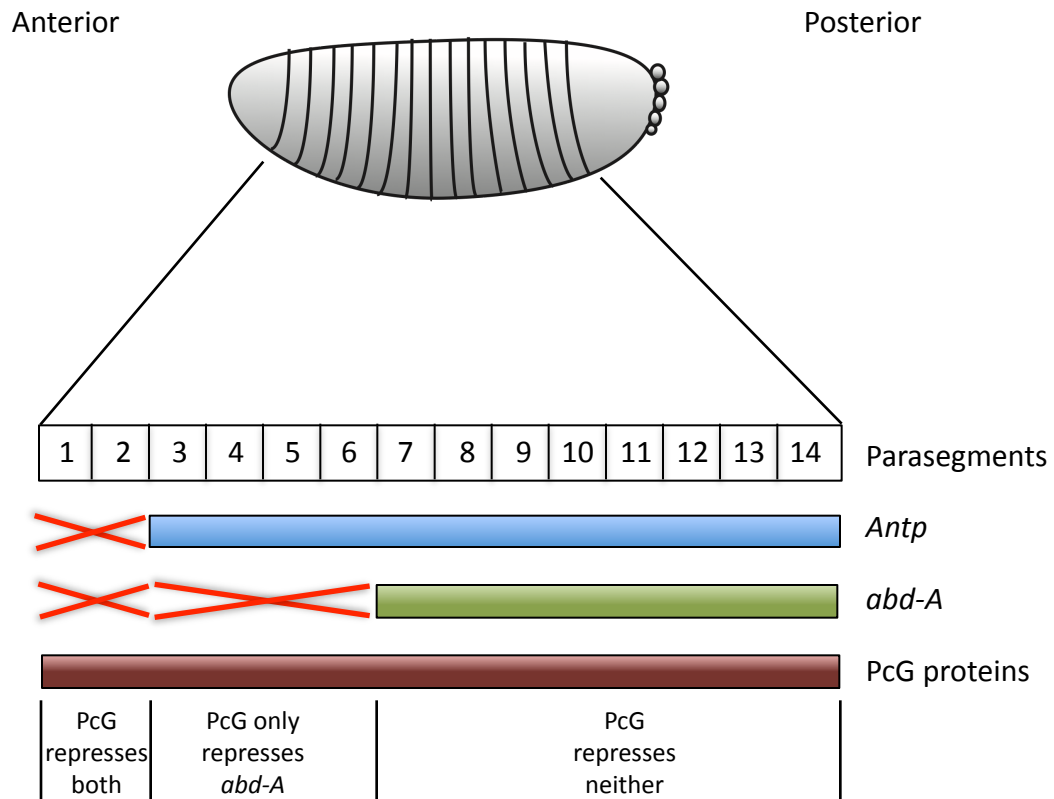
PcG proteins are believed to maintain *Hox* gene expression patterns through heritable (epigenetic) mechanisms. PcG proteins regulate the expression of *Hox* genes during embryogenesis, but they do not create the initial patterns of gene expression along the A-P axis. The segmentation genes, including the *gap* and *pair rule* genes, establish the *Hox* gene expression patterns within the first two hours of embryogenesis (Akam and Martinez-Arias, 1985; Harding and Levine, 1988; Kuziora and McGinnis, 1988; Sanchez-Herrero and Akam, 1989). By 3-4 hours, however the *gap* gene products decay (Gaul et al., 1987; Tautz, 1988), yet the *Hox* gene expression boundaries persist throughout embryogenesis, the larval stage and pupation. This maintenance of gene repression requires the Polycomb Group genes. Several lines of evidence suggest the PcG proteins preserve the expression patterns

dictated by the segmentation genes through a heritable mechanism.

First, in contrast to animals with mutations in *gap* or *pair rule* genes in which proper expression patterns of *Hox* genes are never established, in PcG mutant animals *Hox* gene expression is initiated within the correct spatial boundaries. Misexpression arises later in development, however, indicating these expression patterns fail to be maintained (McKeon and Brock, 1991; Simon et al., 1992; Struhl and Akam, 1985). Second, PcG proteins are ubiquitously expressed in embryos (Paro and Hogness, 1991) yet they repress *Hox* genes only in segments where they were initially silenced. For example the anterior boundary for *abd-A* expression is parasegment<sup>1</sup> 7 (PS7) and that of *Antp* is parasegment 3 (PS3). In parasegments posterior to PS7, both *abd-A* and *Antp* are active, despite the presence of PcG proteins in these parasegments. However, in PS3 through PS6, PcG proteins maintain repression of *abd-A* but *Antp* remains active. In parasegments anterior to PS3, PcG proteins maintain repression of both *Antp* and *abd-A* (Figure 1-1). Thus it appears that PcG proteins can “recognize” the transcriptional status of the gene and only maintain silencing where the gene was originally silenced. This hypothesis is further supported by experiments with transgenes in which developmental stage specific enhancers were combined with DNA elements called Polycomb Response Elements (PREs). PREs direct PcG-dependent silencing of the transgenes, but only in

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<sup>1</sup> The *Drosophila* embryo is divided into 14 parasegments, starting with PS1 at the anterior end. Parasegments are specified by the segmentation genes and are the first evidence of compartmentalization along the A-P axis. The anterior portion of one parasegment and the posterior portion of the parasegment anterior become one segment in the larval and adult stages. Thus parasegments and segments are shifted in register by half a compartment (Martinez-Arias and Lawrence, 1985).



**Figure 1-1. PcG proteins maintain repression of *Hox* genes where initially established.** Expression patterns of two *Hox* genes, *Antp* and *abd-A*, in the *Drosophila* embryo. Despite the presence of PcG proteins in all parasegments, PcG maintains repression only in parasegments where target gene expression was initially repressed by *gap* and *pair rule* genes. Colored bars indicate expression, **X** indicate repression.

regions of the embryos in which the enhancer is initially off (Chan et al., 1994; Poux et al., 1996). How PcG proteins are able to “recognize” the repressed state is unknown but recent data suggest a possible role for histone modifications (Schmitges et al., 2011).

Finally, PcG proteins are required for maintenance of gene repression throughout development. While homozygous PcG mutants die during embryogenesis, the requirement for PcG proteins later in development has been demonstrated in imaginal discs (larval structures that develop into specific body parts of the adult animal). In the imaginal disc clone assay, clones of cells expressing mutant forms of a PcG gene are generated using Flp recombinase-mediated mitotic recombination. *Hox* genes become derepressed within these clones (Beuchle et al., 2001; Birve et al., 2001; Gutierrez et al., 2011; Klymenko et al., 2006; Müller et al., 2002; Scheuermann et al., 2010). Thus PcG proteins are continuously required to maintain expression patterns established in the embryo by other factors.

#### *Biochemical characterization of PcG complexes*

Since their initial genetic discovery, insight into the mechanisms of action of the PcG has been gained through biochemical purifications, which revealed that PcG proteins assemble into multi-protein complexes that have specific functions in altering chromatin structure. These complexes are believed to function together at PcG target genes to create specific chromatin states. Below I describe the known *Drosophila* PcG complexes. In most cases, analogous complexes have been identified

in mammalian cells, and in some cases in other organisms such as *C. elegans* and *Arabidopsis* (reviewed in Hennig and Derkacheva, 2009; Kerppola, 2009; Margueron and Reinberg, 2011; Wenzel et al., 2011).

*Polycomb Repressive Complex 1 (PRC1)*. PRC1 was first purified from *Drosophila* embryos and was shown to include the PcG proteins PC<sup>2</sup>, PSC, PH, dR and substiochiometric amounts of SCM, a number dTAFII proteins and Zeste (Shao et al., 1999). Biochemical experiments indicate that PRC1 binds tightly to DNA and chromatin and can alter their structure (King et al., 2002; Shao et al., 1999). The core functions of PRC1 can be carried out by a PRC1 core complex (PCC) consisting of PSC, PH, PC, and dR (Francis et al., 2001). PCC's *in vitro* activities include inhibition of chromatin remodeling, DNA replication and transcription, and chromatin compaction (Francis et al., 2009; Francis et al., 2004; Francis et al., 2001; King et al., 2002). PCC bound to chromatin can recruit additional nucleosomal arrays from solution (Lavigne et al., 2004) and can physically bridge nucleosomes (Lo et al., manuscript submitted). All of these activities depend on PSC (Francis et al., 2009; Francis et al., 2004; Francis et al., 2001; King et al., 2002; Lo et al., manuscript submitted). The region of PSC responsible for interactions with chromatin is essential for PcG-dependent repression, suggesting that the effects of PRC1 on chromatin are important *in vivo* for gene repression (King et al., 2005).

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<sup>2</sup> A note on nomenclature: Protein products of genes, when denoted by the gene symbol are written in all capital letters. For instance the protein product of the gene *Pc* is PC. When the entire gene name is used only the first letter is capitalized. The protein product of the *Zeste* gene is Zeste.

*PcG Repressive Complex 2 (PRC2).* PRC2 consists of four core proteins: PcG proteins ESC, E(Z), and SU(Z)12; and the chromatin-binding protein NURF-55 (Müller et al., 2002; Ng et al., 2000; Tie et al., 2001). PRC2 was shown to have histone methyltransferase (HMT) activity towards lysine 27 of histone H3, an activity that is required for *Hox* gene silencing *in vivo* (Czermin et al., 2002; Müller et al., 2002).

*dR associated factors (dRAF).* dRAF includes PRC1 members dR and PSC and the F-box protein and demethylase dKDM2. dRAF has E3 ligase activity towards H2A and also harbors H3K36me<sup>3</sup> demethylase activity. H2Aub colocalizes with PRE and promoter regions of the *Ubx* gene and knockdown of dR leads to loss of H2Aub and loss of repression (Wang et al., 2004a), confirming the significance of this activity. Although dRAF and PRC1 share PSC and dR, depletion of PSC or dR but not PC or PH (members of PRC1 but not dRAF) reduces H2Aub levels in S2 cells. This suggests dRAF is primarily responsible for H2A ubiquitination and that PRC1 has an independent function in PcG-dependent repression. This conclusion is supported by *in vitro* assays indicating that dRAF, but not PRC1, can ubiquitinate H2A (Lagarou et al., 2008).

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<sup>3</sup> Histone modifications are denoted by the shorthand: histone, modified residue, modification. Thus H3K36me<sup>2</sup> describes dimethylation (me<sup>2</sup>) of the residue lysine 36 (K36) of histone H3. me=methylation and can be mono (1), di (2) or tri (3). ub=ubiquitination. p=phosphorylation. ac=acetylation.

*Polycomb repressive deubiquitinase (PR-DUB).* The PcG proteins Calypso and ASX comprise the PR-DUB complex, which can deubiquitinate histone H2A *in vitro*. Its activity is required for *Hox* gene repression *in vivo* (Scheuermann et al., 2010). *Asx* and *dR* double mutant embryos show much more severe *Hox* gene misexpression than either mutant alone, suggesting that a balance of H2A ubiquitination and deubiquitination levels is required for repression (Gutierrez et al., 2011).

*Pho repressive complex (PhoRC).* PhoRC includes either one of the only two PcG proteins to exhibit sequence specific binding, PHO (Brown et al., 1998) or Pho-like (PHOL) (Brown et al., 2003), and SFMBT (Klymenko et al., 2006). SFMBT specifically recognizes mono- and di-methylated forms of H3K9 and H4K20 through its MBT domains, although the significance of this recognition for PcG-dependent repression is unknown (Klymenko et al., 2006).

*SXC/Ogt.* A complex containing SXC has not been described but the gene was mapped and found to encode an O-linked *N*-acetylglucosamine (O-GlcNAc) transferase (Ogt), that catalyzes the addition of GlcNAc to proteins, including PH (Gambetta et al., 2009; Sinclair et al., 2009). GlcNAcylated proteins are found at PREs, and are lost in *sxc* mutants (Gambetta et al., 2009).

Thus chromatin at PcG target genes may contain a variety of covalent and non-covalent modifications to chromatin structure and have several PcG complexes



bound. While the requirement for each protein and each modification to maintain gene repression has been demonstrated to some extent, it is unknown whether each modification and protein is required at every target to maintain repression. Genetic studies have shown that many PcG genes are necessary for repression of individual *Hox* genes, thus it is likely at least at some targets many PcG proteins and/or PcG-dependent modifications must be in place in order to maintain repression. It is also largely unknown how each protein or modification contributes to silencing. It is probable that some proteins and/or modifications are more important for gene repression while others play a larger role in recruiting other PcG complexes to target genes, while still others may be responsible for maintaining PcG function through the cell cycle (reviewed in Levine et al., 2004). It should also be noted that the composition and relative importance of the above complexes may not be static—both their compositions and functions may fluctuate through the cell cycle or through development (Kwong et al., 2008; Nègre et al., 2006; Oktaba et al., 2008).

*PcG proteins bind to and regulate many non-Hox gene targets*

Polytene staining revealed the existence of PcG binding sites other than the few intensely studied and well-characterized PREs. Hundreds of targets had been observed on polytene chromosomes for various PcG proteins (Carrington and Jones, 1996; DeCamillis et al., 1992; Lonie et al., 1994; Martin and Adler, 1993; Peterson et al., 1997; Rastelli et al., 1993; Zink and Paro, 1989), and the number of PcG binding

sites has increased with the advent of genome-wide microarray and sequencing technologies. These studies revealed broad binding domains for the histone modification H3K27me3 as well as for PC, with more discrete binding sites for other proteins (Nègre et al., 2006; Schwartz et al., 2006; Tolhuis et al., 2006). Targets generally include transcription factors and developmental regulators. PcG domains were found to comprise a distinctive chromatin 'type' in two large-scale efforts to integrate genome-wide binding data for dozens of chromatin proteins (Filion et al., 2010; Kharchenko et al., 2010; modENCODE Consortium et al., 2010). These functional domains correlate well with physical domains in a genome-wide characterization of chromosomal contacts (Sexton et al., 2012).

Thus PcG proteins have many targets beyond the *Hox* genes whose silencing, it is thought, is dependent upon PcG proteins. Studies of PcG targets in mammalian stem cells suggested a broad role for PcG in regulating developmental programs, and indicated PcG may preferentially bind genes poised for activation later in development (reviewed in Christophersen and Helin, 2010; Jones and Wang, 2010; Pietersen and van Lohuizen, 2008). Studies in different developmental stages and tissues in *Drosophila* also revealed the dynamic nature of PcG target genes (Kwong et al., 2008; Nègre et al., 2006). Thus *Hox* genes may represent one end of the PcG target spectrum where PcG-dependent repression is maintained for the lifetime of the organism, while the other end may include genes that may become expressed later in development.

### *Polycomb Response Elements and targeting of PcG proteins*

In the *Hox* gene clusters and at several other targets PcG proteins work in concert with cis-regulatory sequences called Polycomb Response Elements (PREs). Polycomb Response Elements were initially identified in the BX-C by their ability to confer PcG-dependent maintenance of silencing to *Hox* reporter genes (Chan et al., 1994; Müller and Bienz, 1991; Simon et al., 1993). Polytene chromosome staining and chromatin immunoprecipitation revealed that PREs are binding sites for PcG proteins (Chan et al., 1994; Chiang et al., 1995; Lonie et al., 1994; Orlando et al., 1998; Papp and Müller, 2006; Strutt et al., 1997; Strutt and Paro, 1997; Zink et al., 1991).

It is not completely understood how PcG proteins are recruited to PREs and other binding sites. Except for PHO (Brown et al., 1998) and PHOL (Brown et al., 2003) PcG proteins have not been shown to bind DNA in a sequence-specific manner. PHO has been proposed to recruit other complexes to PREs, and PHO sites have been shown to be required for the function of several PREs (Busturia et al., 2001; Hodgson et al., 2001; Mishra et al., 2001; Tillib et al., 1999). PHO has also been shown to interact with components of PRC1 (Mohd-Sarip et al., 2005; Mohd-Sarip et al., 2006; Mohd-Sarip et al., 2002; Poux et al., 2001) and PRC2 (Poux et al., 2001; Wang et al., 2004b), and the recruitment of various PcG proteins to PREs is dependent on PHO, PHOL or PHO binding sites (Klymenko et al., 2006; Savla et al., 2008; Wang et al., 2004b). However, in *Pho* and *Pho-like* double mutants, localization of PRC1 components and E(Z) to polytene chromosomes is largely

unaffected (Brown et al., 2003). Comparison of genome-wide binding profiles of PHO and PRC1 or PRC2 indicated that while a large amount of overlap exists, not all PRC1 and PRC2 binding sites are accompanied by PHO binding (Beisel et al., 2007; Kwong et al., 2008; Oktaba et al., 2008), indicating that other targeting mechanisms must exist, although a separate study found nearly all PC and PH sites do overlap PHO binding sites (Schuettengruber et al., 2009). Taken together, it is likely PHO and PHOL do play a role in targeting other PcG complexes to chromatin, but it is also likely not the only mechanism.

Another PcG protein, PCL, was shown to be required for E(Z) binding to polytene chromosomes (Savla et al., 2008). As PCL recruitment of E(Z) at the *bxd* PRE occurs through PHO and PHOL (Savla et al., 2008), PCL's targeting effect may be indirect. However, since PHO and PHOL had no effect on E(Z) binding in polytene chromosomes (Brown et al., 2003), PCL may function as the primary targeting protein at some sites.

Many PREs contain short, conserved motifs for DNA binding proteins including GAGA factor (GAF) [also known as Trithorax-like (TRL)] Pipsqueak (PSQ), Zeste, Grainyhead (GRH) [also known as neuronal transcription factor (NTF-1)], DSP1 and SP1/KLF in combination and often with more than one copy of each (Ringrose and Paro, 2007). The requirement for the proteins that recognize these motifs for the function of certain PREs has been demonstrated (Americo et al., 2002; Blastyak et al., 2006; Brown et al., 2005; Brown and Kassis, 2010; Busturia et al., 2001; Dejardin et al., 2005; Hodgson et al., 2001; Horard et al., 2000; Huang et al., 2002;

Mahmoudi et al., 2003; Mishra et al., 2001; Mulholland et al., 2003; Schweinsberg and Schedl, 2004; Schwendemann and Lehmann, 2002) and GAF has been shown to facilitate binding of PHO to chromatin (Mahmoudi et al., 2003), but it is unclear whether any one of these factors has a role in the general recruitment of PcG complexes. Comparison of genome-wide binding profiles of DSP1 and GAF with those of PC and PH showed roughly 50% colocalization (Schuettengruber et al., 2009), and a smaller study also found significantly different binding patterns for GAF and PC and PH (Nègre et al., 2006).

It has been proposed that the histone modification H3K27me3 deposited by PRC2 serves to recruit PRC1 to target sites, as H3K27me3 is recognized by the chromodomain of PC (Cao et al., 2002; Fischle et al., 2003b; Min et al., 2003; Wang et al., 2004b). Several lines of evidence indicate that H3K27me3 can target PRC1 to chromatin. Swapping of the chromodomain of the protein HP1 with the chromodomain of PC serves to target the chimeric protein to PC binding sites, however it also binds heterochromatin, where HP1 is normally bound (Platero et al., 1995). In human cells knockdown of the demethylase for H3K27 di- and trimethylation leads to greater levels of PRC1 at *Hox* genes (Lee et al., 2007), and targeting of H3K27me3 by a viral SET domain in the absence of endogenous H3K27me3 leads to recruitment of PRC1 at target promoters in HeLa cells (Mujtaba et al., 2008). Evidence does exist, however, against a role for H3K27me3 in targeting. Knockdown of PCL reduces levels of H3K27me3, albeit modestly, but does not result in a decrease in PH binding to target sites (Nekrasov et al., 2007).

Importantly genome-wide analysis in *Drosophila* shows broad domains of H3K27me3 covering target genes with H3K27me3 depletion at PREs, where PcG proteins are bound (Schuettengruber et al., 2009; Schwartz et al., 2006). Many other studies also give evidence to support the nucleosome-depletion and/or high turnover at PREs (Deal et al., 2010; Kahn et al., 2006; Mishra et al., 2001; Mito et al., 2007; Mohd-Sarip et al., 2006). It is difficult to reconcile the targeting of PcG proteins to discrete sites (discrete binding sites are exhibited by many PcG proteins other than PC) by a mark that is simultaneously spread often over tens of kilobases and depleted and/or rapidly turning over at the sites to which it supposedly target the proteins.

If H3K27me3 indeed serves as a method of recruitment of PRC1, it is unlikely to be the only method. Evidence exists for direct targeting of PRC1 by PHO (Mohd-Sarip et al., 2005; Mohd-Sarip et al., 2006; Poux et al., 2001), as discussed above. Additionally, the overlap of H3K27me3 and PC on polytene chromosomes is not complete (Ringrose et al., 2004). In mammalian systems several studies report the function of PRC1 in the absence of PRC2 (Schoeftner et al., 2006; Tavares et al., 2012; Vincenz and Kerppola, 2008; Yu et al., 2012).

In mammalian systems there is evidence that PRC2 can be recruited to chromatin by noncoding RNAs (ncRNAs). EZH2 has been shown to bind to a ncRNA called *RepA*, a short ncRNA within the larger *Xist* ncRNA that plays a role in X chromosome inactivation. EZH2 binding to RepA serves to recruit PRC2 to the inactive X chromosome (Zhao et al., 2008). Similarly, EZH2 and SU(Z)12 were shown to interact with a long ncRNA termed *HOTAIR*, that is transcribed from the *HOXC*

locus and silences the *HOXD* locus in trans. Loss of HOTAIR leads to loss of H3K27me3 and SU(Z)12 binding at the *HOXD* locus, suggesting *HOTAIR* targets EZH2 and SU(Z)12 to the locus (Rinn et al., 2007; Tsai et al., 2010). EZH2 and SU(Z)12 were also shown to interact with the ncRNA *kcnq1ot1* from the *Kcnq1* cluster of paternally imprinted genes. PRC2 is required for imprinted silencing of the *Kcnq1* locus and *kcnq1ot1* is required for H3K27 trimethylation of the *Kcnq1* locus, suggesting a role for *kcnq1ot1* in targeting PRC2 at the *Kcnq1* cluster (Pandey et al., 2008; Terranova et al., 2008). Additionally genome-wide identification of RNA bound to EZH2 in embryonic stem cells found >9,000 RNAs, 216 of which were long intergenic ncRNAs and 34 of which are from imprinted clusters of genes. The role of PRC2 in silencing one such imprinted locus *DIK1-Gt/2* and the role of the ncRNA *Gt/2* in targeting PRC2 to the locus was shown (Zhao et al., 2010). Thus targeting of PRC2 by ncRNA may have a broad role in mammalian cells.

No such examples of ncRNA targeting of PRC2 has been described in *Drosophila*, indicating that this mechanism of targeting may not be universal. It should be noted though that it has been suggested that ncRNAs transcribed from the *Ubx*-regulating *bxd* PRE may serve to recruit ASH1, a histone methyltransferase and trxG member. The transcription of these ncRNAs correlates with binding of ASH1 and *Ubx* transcription, ASH1 can bind these *bxd* ncRNAs *in vitro* and binding of ASH1 to the *bxd* locus seems to be dependent on RNA-DNA hybrids (Sanchez-Elsner et al., 2006). (Although a separate study did not observe ASH1 binding at the *bxd* PRE in either the repressed or active state of *Ubx* (Papp and Müller, 2006)). As

transcription through a PRE has been shown to be able to cause a switch from repression to activation, which is then maintained (Cavalli and Paro, 1998; Cavalli and Paro, 1999; Hogga and Karch, 2002; Rank et al., 2002; Schmitt et al., 2005), it has been proposed that continual transcription through PREs is required for maintenance of activation. It has also been suggested that the persistence of R-loops (RNA bound to one strand of a melted DNA duplex) may direct memory of activation through mitosis (Ringrose and Paro, 2007).

Understanding the mechanisms of recruiting PcG proteins to targets may be key to understanding how they are maintained through the cell cycle. Since most PcG proteins do not bind with sequence specificity, the disruption of their binding to chromatin during the cell cycle may be particularly problematic. In the case of their displacement from chromatin they must not only be directed back to their target binding sites, but their function must be re-established in a tissue-specific manner. A clearer understanding of the mechanisms of PcG targeting may shed light on this matter.

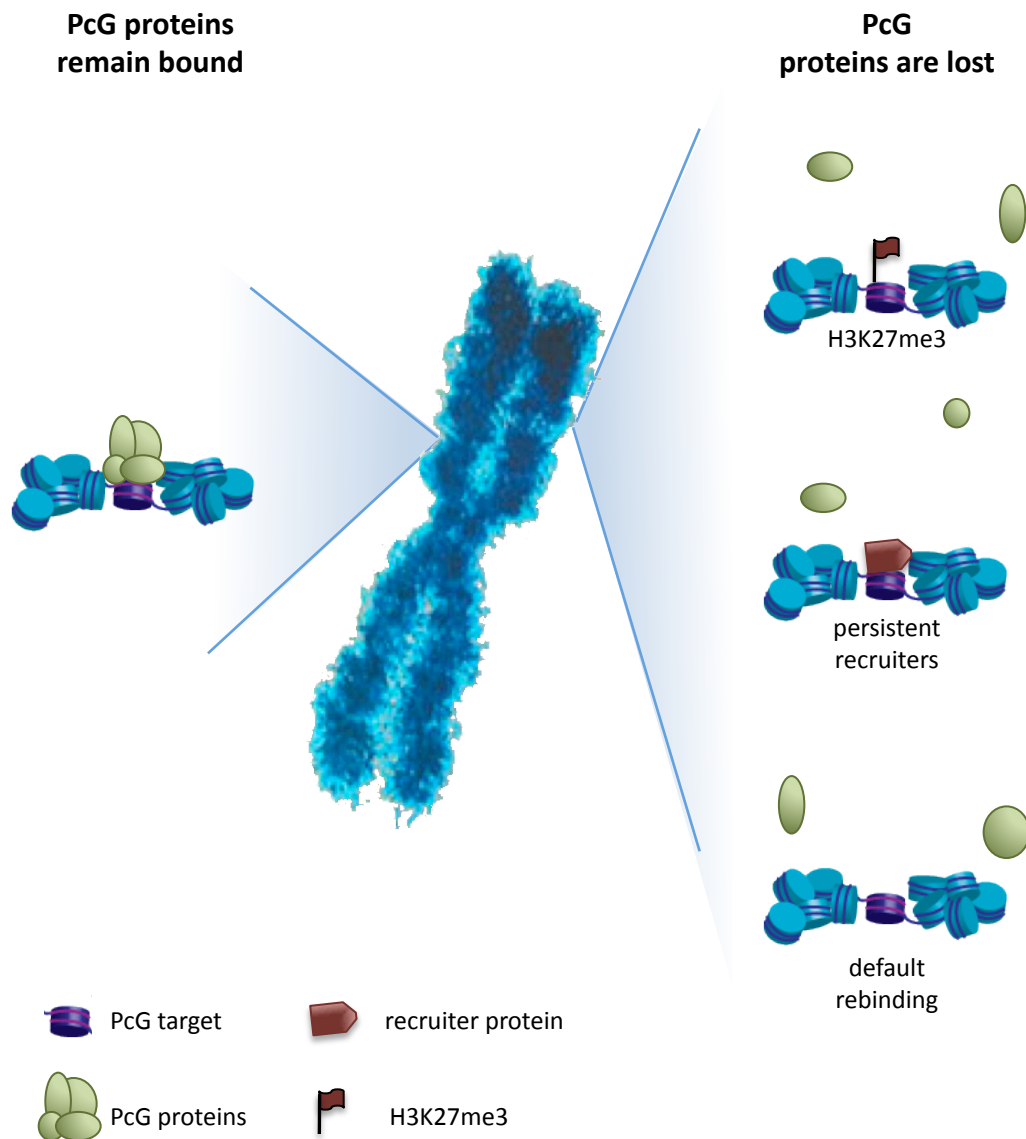
#### *Inheritance of PcG-dependent repression through mitosis*

Any chromatin-based mechanism of epigenetic inheritance must contend with the potentially disruptive events that occur during mitosis, including large-scale physical and biochemical changes and transcriptional repression. It is unknown how PcG-dependent repression is maintained through mitosis. Two models can be envisioned to explain how such repression might be propagated through mitosis,



based on the behavior of PcG proteins during mitosis. In one model PcG proteins remain bound to mitotic chromosomes, and thus they may constitute the memory themselves. In the second model PcG proteins are lost from chromatin during mitosis. In this case transcriptional memory must reside elsewhere, such as in the altered chromatin structures induced by PcG proteins, so that the proteins may be re-established upon mitotic exit (Figure 1-2).

Some data exist for the persistence of PcG proteins through mitosis, while other data exist for the dissociation of PcG proteins from mitotic chromosomes. All such localization has been determined by immunostaining. In *Drosophila* embryos PSC was found to be localized to mitotic chromosomes (Martin and Adler, 1993) and also to be largely lost from them, with only an estimated 5-7% remaining in mitosis (Buchenau et al., 1998). Similarly in larval neuroblasts PC was shown by one study to remain bound to mitotic chromosomes (Fanti et al., 2008) and by a second to be largely lost from them (Dietzel et al., 1999). Such differences may be explained by differences in experimental treatment of the samples (fix conditions, for example), but leave the question of what happens to PcG proteins during mitosis unanswered. Other studies of PcG proteins in mitosis give similarly incongruent answers in different developmental stages, which could be attributed to real differences in mitotic localization at different developmental stages, or it could also be explained by differences in experimental details. PC was seen to be largely lost from mitotic chromosomes in embryos (Buchenau et al., 1998; Dietzel et al., 1999), but a



**Figure 1-2. Models for inheritance of PcG-dependent repression through mitosis.** Models for the inheritance of PcG-dependent repression through mitosis can be grouped into two classes: those in which PcG proteins remain bound to mitotic chromosomes (left) and thus may constitute the memory themselves, and those in which PcG proteins are lost from mitotic chromosomes (right). If PcG proteins are lost from mitotic chromosomes they may leave a 'mark' to facilitate rebinding at target genes upon mitotic exit. This mark may take the form of the histone modification H3K27me3 (right, top) or the persistence of proteins thought to recruit PcG proteins to target genes (right, middle). If PcG proteins bind targets by default, no mark may be necessary (right, bottom).

significant fraction was seen to be retained in S2 cells in a separate study (Messmer et al., 1992). PH was found to remain bound in larval neuroblasts (Fanti et al., 2008), but another study showed it is lost in embryos, with < 2% of PH remaining associated with metaphase chromosomes (Buchenau et al., 1998). PHO and E(Z) were also shown to remain bound to mitotic chromosomes (Fanti et al., 2008). Again, it is unclear if these observed differences reflect real differences in mitotic localization or not, as separate studies in the same developmental tissue gave seemingly conflicting data. As such, the behavior of PcG proteins in *Drosophila* during mitosis is still largely an open question.

Some data exists for the retention of mammalian PcG proteins on mitotic chromatin. Homologs of components of both PRC1 and PRC2 have been found to colocalize with mitotic chromatin to some extent in various cell lines and tissues, mainly by immunofluorescence (Aoto et al., 2008; Elderkin et al., 2007; Fang et al., 2004; Hansen et al., 2008; Hernández-Muñoz et al., 2005; Mak et al., 2002; Miyagishima et al., 2003; Saurin et al., 1998; Suzuki et al., 2002; Vincenz and Kerppola, 2008; Voncken et al., 1999). The histone modification H3K27me3 was also found to persist in mitosis (Aoto et al., 2008). No information, however, exists as to whether mammalian PcG proteins remain bound to target genes during mitosis.

If PcG proteins are lost from mitotic chromatin, several models have been proposed to account for the maintenance of PcG-dependent repression. The histone modification H3K27me3 has been suggested as a candidate for directing epigenetic memory (Fischle et al., 2003b; Wang et al., 2004b). For H3K27me3 to

direct epigenetic memory it must fulfill the following criteria: 1) it must be able to target PcG proteins to marked genes and 2) it must persist through mitosis.

Evidence for fulfillment of the first criterion is inconclusive, as discussed above. As for the second criterion, evidence exists in mammalian cells for the maintenance of H3K27me3 on mitotic chromosomes (Aoto et al., 2008), but has not been shown in *Drosophila*.

In the absence of PcG proteins on mitotic chromatin, if non-PcG proteins that are thought to play a role in recruiting PcG proteins to chromatin (such as GAF, PSQ, etc. as discussed above) persist at target sites in mitosis, they may allow the re-recruitment of PcG proteins to those targets upon mitotic exit. GAF and PSQ have been reported to remain on mitotic chromosomes (Schwendemann and Lehmann, 2002), although one report describes the movement of GAF from euchromatic sites to different heterochromatic sites during mitosis (Platero et al., 1998). The mitotic behavior of other non-PcG targeting proteins is unknown. Given that it is doubtful that these recruitment proteins generally target PcG proteins (see discussion above) it is also unlikely that they play a general role in transmission of mitotic memory.

Another model how PcG-mediated repression is maintained through mitosis is informed by the idea that the default state of PREs is the repressed state (Müller and Kassisi, 2006; Ringrose and Paro, 2007). This implies that PREs are bound by PcG proteins by default. Thus if PcG proteins are lost from targets during mitosis they will automatically rebind, and so it follows that only the active state must be marked during mitosis. Arguments for repression being the default state include studies in

which a PRE placed within various reporter gene constructs can silence heterologous enhancers and promoters (Dellino et al., 2004; Sengupta et al., 2004). In imaginal disc clones, loss of trxG proteins led the repression of *Ubx* by PcG proteins (Klymenko and Müller, 2004), and several other studies indicated that transcription through a PRE could lead to switching its state to activation (Cavalli and Paro, 1998; Cavalli and Paro, 1999; Hogga and Karch, 2002; Rank et al., 2002; Schmitt et al., 2005), lending the idea that PREs are silenced unless transcription disrupts this activity. However, evidence exists that argues against default binding of PcG proteins to PREs. Several studies following PcG targets in different developmental tissues or cell lines indicate that PcG targets can exist in a “null” state. In this state the gene, which is PcG-bound in other tissues, is devoid of PcG proteins or trxG proteins and is inactive (Kwong et al., 2008; Schwartz et al., 2010). These “null” state targets do not bind PcG proteins, even though trxG proteins, which are thought necessary to counteract PcG binding, are not present. Thus if binding of PcG targets is not automatic, then some mechanisms must exist to facilitate their rebinding if they are lost during mitosis.

## **Mitosis and chromatin**

How PcG proteins and other chromatin binding proteins behave during mitosis is presumably driven in part by the biochemical and physical changes to chromatin that occur during mitosis.

One large-scale change that occurs during mitosis is the loss of compartmentalization that results from the disassembly of the nuclear envelope during 'open' mitoses, which many animal cells undergo. In contrast many fungi and yeast undergo 'closed' mitosis in which the nuclear envelope remains intact. It is possible that chromatin proteins dissociate from mitotic chromatin due to the increase in volume and corresponding decrease in their concentration that accompanies open mitosis. *Drosophila* undergoes 'semi-closed' mitosis. Remnants of the nuclear membrane persist at least through metaphase. This semi-closed mitosis has been observed during embryogenesis in the late syncytial blastoderm (Paddy et al., 1996; Stafstrom and Staehelin, 1984), in larval neuroblasts (Katsani et al., 2008) and in Kc cells (Debec and Marcaillou, 1997). This manner of mitosis may have implications for chromatin-bound factors involved in transcriptional memory that are released during mitosis. The compartmentalization that remains during semi-closed mitosis may limit the diffusion of these factors from the mitotic chromatin.

At the level of higher order chromatin structure, the changes that take place during mitosis are drastic. From interphase to mitosis chromatin is compacted roughly 2-fold in yeast to ~4-50 fold in mammalian cells, which translates to a linear compaction of ~160-fold in yeast to ~10,000-20,000 fold in mammalian cells (reviewed in Belmont, 2006). It is possible that these structural changes may physically preclude binding of chromatin proteins, and an early hypothesis for the cause of transcriptional silencing during mitosis (discussed below) was decreased

accessibility of mitotic chromosomes to the transcriptional machinery due to condensation (Johnson and Holland, 1965). However, a later study showed that mitotic chromosomes remain accessible to transcription factors and chromatin structural proteins (Chen et al., 2005).

Another change to chromatin that occurs during mitosis that may alter the binding of chromatin proteins is the global phosphorylation of Ser10, Ser28, Thr3 and Thr11 residues of histone H3. These residues are close in proximity to other residues of the protein that are covalently modified, and it has been found that the mitotic phosphorylation of these Ser and Thr residues can interfere with binding of factors that recognize nearby covalent modifications. This mechanism has been described as a 'methyl/phos switch' or 'binary switch' (Fischle et al., 2003a). One example is given by differential binding of HP1 to singly vs. doubly modified H3. HP1 binds to the H3K9me3, a modification associated with heterochromatin. HP1 binding is greatly reduced upon phosphorylation of H3S10, as it binds poorly to the doubly modified histone tail, leading to its dissociation from mitotic chromosomes (Fischle et al., 2005; Hirota et al., 2005).

During mitosis transcription is largely shutdown, which was first demonstrated by the failure of mitotic cells or mitotic extracts to incorporate radioactive NTPs (Johnson and Holland, 1965; Prescott and Bender, 1962). The mechanism of transcription shutdown involves both inactivation of the transcription machinery and/or exclusion from mitotic chromosomes (reviewed in Gottesfeld and Forbes, 1997). RNA polymerase III (Gottesfeld et al., 1994; Leresche et al., 1996;

White et al., 1995), RNA polymerase II (Leresche et al., 1996; Long et al., 1998) and TFIID (Segil et al., 1996) are inactivated by phosphorylation, and TFIIA and TFIIB (Segil et al., 1996) and transcription elongation complexes are excluded from mitotic chromosomes (Parsons and Spencer, 1997). Transcription termination factor 2 (TTF2), a RNA polymerase I and II termination factor, goes to chromatin in mitosis and reduces the amount of the elongating form of Pol II (Jiang et al., 2004), supporting a role for TTF2 in mitotic repression of transcription.

Many sequence specific transcription factors are lost from chromatin or are inactivated by phosphorylation during mitosis. Myc and myb are phosphorylated at mitosis, which may correlate with loss of binding (Luscher and Eisenman, 1992). Sp1 and sp3 (He and Davie, 2006), HSF1, C/EBP Oct-1, Oct-2, Ets-1, B-Myb, c-Fos, E2F-1 and Bcl-6 are all excluded from mitotic chromosomes by an unknown mechanism (Martinez-Balbas et al., 1995). Transcription factors of the C2H2 zinc finger family, including the protein Ikaros, are phosphorylated in the highly conserved linker between zinc-finger domains during mitosis which causes loss of DNA binding activity (Dovat et al., 2002). The chromatin remodeler Swi/snf has been shown to be inactivated by phosphorylation (Sif et al., 1998) and excluded from mitotic chromosomes during mitosis (Muchardt et al., 1996). In addition histone deacetylases (HDACs) and histone acetyltransferases (HATs) are lost from mitotic chromosomes (Kruhlak et al., 2001).

The shutdown of transcription during mitosis necessitates the restart of transcription after the conclusion of mitosis. As each cell contains genes that are



expressed and those that are repressed, if the transcriptional profile of the parent cell is to be maintained in the daughter cells, the genes that were expressed in the parent must be re-expressed in the daughter cells and likewise, genes that were repressed need to remain repressed. The loss of both sequence specific and general transcription factors leads to a conundrum: how does a pattern of gene expression get restored after mitosis if the factors that lead to transcription are removed?

While less studied, a similar condition may hold true for repressed genes. If factors required for repression are lost from mitotic chromosomes, how is the pattern of gene repression restored upon mitotic exit? Several ideas have been posited on the mechanism of restoration of transcription after mitosis. One idea is that the concentration of binding factors and their affinity for binding sites directs their rebinding during the subsequent G1 phase. Another idea is the existence of mitotic bookmarks to facilitate the restart of transcription at formerly active genes. The same may be true for repressed genes.

### **Mitotic bookmarking**

A set of phenomena has been described that has been proposed to serve as a memory of active transcriptional status and to allow the restart of transcription of active genes upon mitotic exit. These phenomena, known as mitotic bookmarking, involve a placement of a molecular feature or “bookmark” that persists through mitosis. Many molecular features have been demonstrated to persist in mitosis, making them potential mitotic bookmarks. These include general transcription

factors, sequence specific transcription factors, nuclease and chemical hypersensitivity indicative of altered chromatin structures, histone modifications and chromatin binding proteins (Sarge and Park-Sarge, 2009). While many candidate bookmarks in mitosis have been identified, the mechanism by which they affect the restart of transcription after mitosis and the extent to which their activities are essential for transcription in G1 have been addressed in fewer cases.

#### *Chemical and nuclease sensitivity*

Early evidence of mitotic bookmarks involved persistence of nuclease or chemical sensitivity, indicative of altered chromatin structures. Hypersensitivity to the nuclease DNaseI, and increased sensitivity to permanganate, indicative of single-stranded DNA, was observed to persist in mitosis at active or inducible gene promoters in several studies (Gazit et al., 1982; Martínez-Balbás et al., 1995; Michelotti et al., 1997). While not demonstrated, it could be imagined that altered chromatin structures may promote the restart of transcription after mitosis by preferential recruitment of the transcription machinery due to greater accessibility. Alternatively it is possible that the increased or persistent chemical and nuclease sensitivity is indicative of persistent protein binding that contributes to transcriptional reactivation.

#### *Transcription factor binding*

Indeed evidence for the persistence of DNA binding proteins on mitotic chromatin exists for a number of instances of mitotic bookmarking, including several of the genes exhibiting nuclease or chemical hypersensitivity. Both general and sequence-specific transcription factors were shown to persist at gene promoters. Persistent factors include TBP, TAF<sub>II</sub>100, TFIID (Christova and Oelgeschläger, 2002; Xing et al., 2008), HSF2 (Xing et al., 2005), Foxl1 (Yan et al., 2006) and Runx2 (Young et al., 2007a; Young et al., 2007b; Zaidi et al., 2003). Persistence of binding of general transcription factors likely facilitates the reassembly of transcription-competent transcription initiation complexes at the conclusion of mitosis.

Additional mechanistic details were discovered for mitotic bookmarking by TBP and HSF2, linking transcription factor binding and altered chromatin structure. Both were shown to interact with the protein phosphatase PP2A, which can dephosphorylate the condensin subunit CAP-G, thus inactivating it (Xing et al., 2008; Xing et al., 2005). This inactivation was suggested to prevent local chromatin compaction at the promoter, which may facilitate reassembly of the transcription machinery at the end of mitosis.

The requirement for a transcription factor during mitosis for the inheritance of gene expression was demonstrated in a study of the transcription factor HNF-1 $\beta$ , a transcription factor required for the expression of several renal cystic genes. HNF-1 $\beta$  was shown to bind to mitotic chromosomes. Significantly, in the absence of HNF-1 $\beta$ , its target genes continue to be expressed in non-proliferating cells, but when cells are induced to proliferate by ischemia-reperfusion injury some target genes

lose activation, consistent with a specific requirement for HNF-1 $\beta$  during mitosis for continued activation of those target genes in progeny cells (Verdeguer et al., 2010).

### *Histone modifications*

While a large number of histone modifications have been shown to persist through mitosis (Garcia et al., 2005; Kouskouti and Talianidis, 2005; Valls et al., 2005; Xin et al., 2007; Young et al., 2007b; Zaidi et al., 2003), the requirement for these modifications during mitosis or their role in directing transcription at the end of mitosis or even during the rest of interphase is unclear. The significance of two such modifications for mitotic bookmarking and transcription in progeny cells, however, has been demonstrated.

By live-cell imaging it was found that daughter cells in *Dictyostelium* inherit patterns of transcription of a single gene from the parent cell, as defined by pulse length and pulse rate of transcription of that gene. This inheritance depends on the H3K4 HMTs Set1 and Ash2, and the K4 residue of H3, indicating that the transcriptional memory is mediated through this histone modification, which persists in mitosis. Interestingly however, while *patterns* of transcription of single genes are lost with the loss of H3K4 methylation, the *state* of transcriptional activity, i.e. active expression, is not (Muramoto et al., 2010). The functional consequence of loss of transcription patterns of single genes while the expression state remains the same is unclear.

The histone modification H4K5ac is bound by the bromodomain containing protein BRD4, both of which have been shown to persist during mitosis (Dey et al., 2003; Dey et al., 2000; Nishiyama et al., 2006). BRD4 is required for the transcription of genes expressed exclusively in early G1, highlighting its role as a mitotic bookmark. The reduction of early G1 transcription due to BRD4 knockdown leads to apoptosis, underscoring the importance of this process (Dey et al., 2009; Mochizuki et al., 2008; Yang et al., 2008). BRD4 and H4K5ac is also involved in the rapid reactivation of an inducible reporter gene locus after mitosis (Zhao et al., 2011). There is evidence that both decompaction of chromatin (Zhao et al., 2011) and recruitment of P-TEFb, a kinase that phosphorylates Ser2 of the C-terminal domain of Pol II, promoting transcriptional elongation (Dey et al., 2009), contribute to mitotic bookmarking by BRD4.

The histone modification H3K9me3, associated with heterochromatin, is one example of a mitotic bookmark for the repressed state. H3K9me3 persists through mitosis (Peters et al., 2002) and is recognized by the chromodomain protein HP1 (Bannister et al., 2001; Lachner et al., 2001). Induced heterochromatinization of a reporter gene can cause repression that is epigenetically inherited (Nakayama et al., 2000), as is the heterochromatin at the mating type loci and centromeric regions in yeast (reviewed in Grewal and Moazed, 2003). While HP1 binding to mitotic chromatin is reduced by the phosphorylation of H3S10 as discussed above, the persistence of H3K9me3 can redirect binding of HP1 upon mitotic exit.

### *Other examples*

In addition to contributing to the transmission of transcriptional status from mother to daughter cell so that stable cell phenotypes can be maintained, it is posited that a second function of mitotic bookmarking is to poise genes for expression early in G1 (Sarge and Park-Sarge, 2009). This seems to be the case for BRD4 and H4K5ac, as discussed above. Another mitotic bookmark shown to be required for rapid re-activation of genes upon mitotic exit is the mammalian protein mixed lineage leukemia (MLL). The binding pattern of MLL changes during mitosis and it binds the promoters of highly expressed genes specifically in mitosis (Blobel et al., 2009).

The zinc finger protein CTCF, shown to function at many insulator/boundary elements and to mediate long-range interactions, is retained on mitotic chromosomes. Significantly it was demonstrated that the long-range interactions mediated by CTCF also persist through mitosis demonstrating that higher-order chromatin structure can be maintained through mitosis (Burke et al., 2005), and suggesting that elements responsible for organizing the genome into separate active and inactive domains may play a role in transcriptional memory.

The histone variant H2A.Z, which binds at the +1 nucleosome position at many active genes, was found to shift to cover the TSS during mitosis. As nucleosomal occupancy at the TSS is incompatible with transcription, this may be a mechanism to shut transcription off during mitosis, but the retention of H2A.Z at

active genes may also be a mechanism for reactivation after mitosis (Kelly et al., 2010).

## **Summary and hypothesis**

Stable patterns of gene expression are required for normal development and are maintained by epigenetic mechanisms. Mechanisms of epigenetic inheritance are thought to involve changes in chromatin structure. If such chromatin structures underlie epigenetic inheritance and thus are heritable, they must be maintained through mitosis or re-established upon mitotic exit. Mitosis involves many physical and biochemical changes, including general transcriptional repression that may be disruptive to a chromatin-based mechanism of inheritance. Several studies have investigated how an active state may be propagated through mitosis, but there are fewer studies of propagation of repressive states through mitosis. The Polycomb Group proteins are model system for epigenetic regulation through modification of chromatin structure. How PcG-dependent repression is propagated through mitosis is unknown but is central to understanding how these proteins mediate epigenetic effects.

I hypothesize that PcG proteins remain bound to mitotic chromosomes and are thus able to reform repressive structures upon mitotic exit. To test this idea, the behavior of a number of PcG proteins during mitosis was examined via several methodologies, including immunofluorescence, biochemical fractionation, chromatin immunoprecipitation and chromatin immunoprecipitation followed by

genome-wide sequencing. These studies provide insight into how PcG-dependent repression is maintained through mitosis and may have implications more broadly for other instances of epigenetic memory.



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## **Chapter 2**

**PcG proteins remain associated with chromatin during mitosis**

## Introduction

Epigenetic mechanisms mediate stable inheritance of gene expression patterns through multiple rounds of cell division, which is crucial for the normal development of multi-cellular organisms. One class of epigenetic mechanisms is believed to involve heritable changes to chromatin structure at target genes. The hypothesis that chromatin structures are the basis of epigenetic inheritance implies that these structures can be propagated through cell division. This raises the question of how specific chromatin structures that may carry information about gene regulation can be maintained during or re-established after mitosis, a process that alters chromatin structure and is accompanied by many biochemical changes including general transcription repression. The Polycomb Group proteins of *Drosophila melanogaster* are an example of factors that use chromatin-based mechanisms for epigenetic regulation of gene expression and are thus a model system for addressing mechanisms of epigenetic inheritance through mitosis.

Polycomb Group (PcG) proteins are required to maintain gene silencing during development and in differentiated cells (reviewed in Pirrotta, 1998; Ringrose and Paro, 2004; Simon and Tamkun, 2002). PcG proteins assemble into multi-protein complexes with an array of effects on chromatin (reviewed in Muller and Verrijzer, 2009; Simon and Kingston, 2009). One complex, Polycomb Repressive Complex 1 (PRC1) inhibits transcription, DNA replication, and chromatin remodeling *in vitro*, and can compact chromatin (Francis et al., 2009; Francis et al., 2004; Francis et al., 2001; King et al., 2002). Members of PRC1 are present in a second complex,

dRAF, which is an E3 ligase for ubiquitination of histone H2A (Lagarou et al., 2008). PR-DUB is a PcG complex that de-ubiquitinates histone H2A (Scheuermann et al., 2010); and both E3 ligase activity and deubiquitinase (DUB) activity are important for gene silencing (Gutierrez et al., 2011; Scheuermann et al., 2010; Wang et al., 2004a). PRC2 has methyltransferase activity, and uses lysine 27 of histone H3 as a major substrate (producing H3K27me3). Components of both PRC1 and PRC2 can interact with H3K27me3, suggesting this modification may play a central role in recruiting or retaining PcG complexes (Margueron and Reinberg, 2011). The PhoRC complex contains the sequence specific DNA binding protein pleiohomeotic (PHO), and a protein that recognizes specific modification states of histones (Klymenko et al., 2006). Finally, the PcG protein SXC is the major enzyme for addition of O-linked sugar groups to proteins (Gambetta et al., 2009). Thus, PcG regulated genes likely have a unique constellation of histone and protein modifications, several PcG proteins tightly bound to them and a locally altered chromatin structure (Mishra et al., 2001). PcG-dependent chromatin states are hypothesized to be heritable, and thus to mediate stable gene silencing established by transient regulatory events (reviewed in Schwartz and Pirrotta, 2007).

The extensive biochemical characterization of PcG proteins has not yet provided insight into how PcG-dependent repression can be propagated through mitosis. One model is that PcG proteins remain bound to mitotic chromosomes through mitosis. An alternative model is that PcG proteins are released from mitotic chromosomes but that certain proteins or chromatin features mark their binding

sites through mitosis to allow re-establishment PcG protein binding after mitosis (Francis and Kingston, 2001). Some transcriptional regulators have been shown to persist on mitotic chromosomes to facilitate reactivation of genes in G1, in a phenomenon termed “mitotic bookmarking” (reviewed in Delcuve et al., 2008; Zaidi et al., 2011). In other cases, modification of chromatin or transcription regulators disrupts their binding in mitosis (Dovat et al., 2002; Fischle et al., 2005; Hirota et al., 2005). For PcG proteins in *Drosophila*, evidence from different immunofluorescence studies and for different proteins supports both retention on mitotic chromosomes and loss from them (Beck et al., 2010; Buchenau et al., 1998; Fanti et al., 2008; Martin and Adler, 1993), leaving the fundamental distinction between the two models unresolved.

To understand how PcG-dependent repression can be maintained through mitosis the extent to which PcG proteins associate with mitotic chromosomes was examined using two methods, immunofluorescence and biochemical fractionation, in *Drosophila* tissue culture cells.

## **Results**

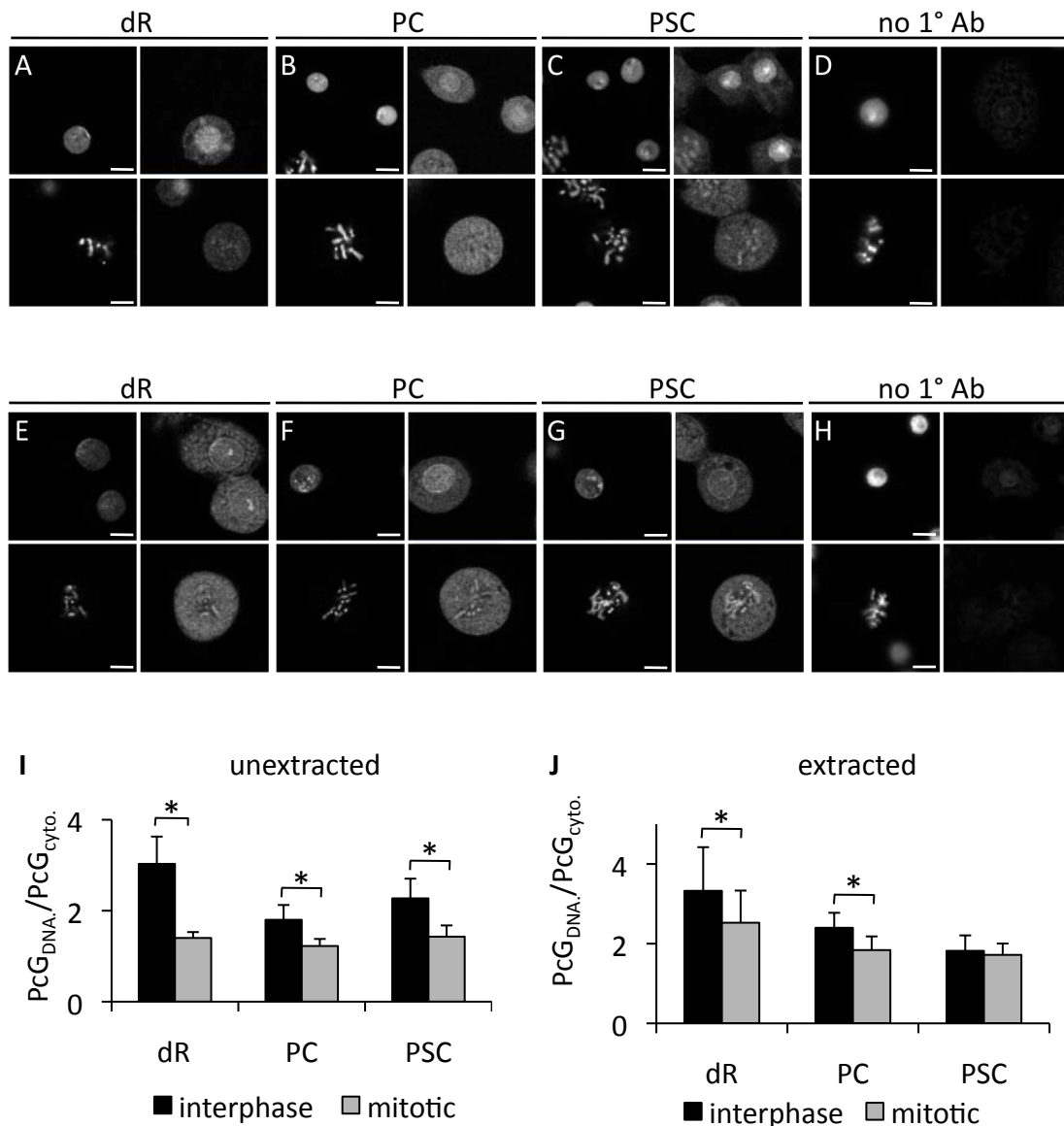
*Polycomb Group proteins are not excluded from mitotic chromosomes.*

To characterize PcG protein binding to mitotic chromosomes, immunofluorescence with antibodies against Polycomb (PC), PSC and dRING (dR) was carried out in *Drosophila* S2 cells. S2 cells are a well-characterized cell line derived from embryos. The extent of immunostaining for PcG proteins that

colocalizes with the DNA, as determined by Hoechst staining, was compared with PcG protein staining in the cytoplasm. All three proteins display mainly nuclear localization in interphase cells, as expected (Figure 2-1A-C, top panels). In mitotic cells, each protein is distributed throughout the cell body, and is neither restricted to nor excluded from chromosomes (Figure 2-1A-C, bottom panels). The PcG signal overlapping nuclei (control) or chromosomes (mitotic) and PcG signal in the cytosol were measured, and the average intensity of each was compared in mitotic and control cells. This quantification confirms a persistent but decreased PcG protein signal that colocalizes with chromatin in mitotic cells (Figure 2-1I). To ask if PcG proteins are more loosely associated with chromatin in mitotic cells, cells were extracted with detergent prior to fixation. This method has been used previously to identify tightly bound chromatin proteins (Rowbotham et al., 2011). Detergent-extracted cells do not show reduced colocalization of PC or dR with mitotic chromosomes, and colocalization of PSC with chromatin relative to the cytoplasm is actually increased (Figure 2-1E-G, J). This could be explained by extraction of cytosolic protein by the detergent. Thus PcG proteins are not excluded from mitotic chromosomes in S2 cells.

*Polycomb Group proteins fractionate with chromosomes in G2/M cells.*

Immunofluorescence results can be highly dependent on fixation conditions and antigen accessibility. Thus an independent means was used to test whether PcG proteins associate with mitotic chromosomes. Biochemical fractionation, followed



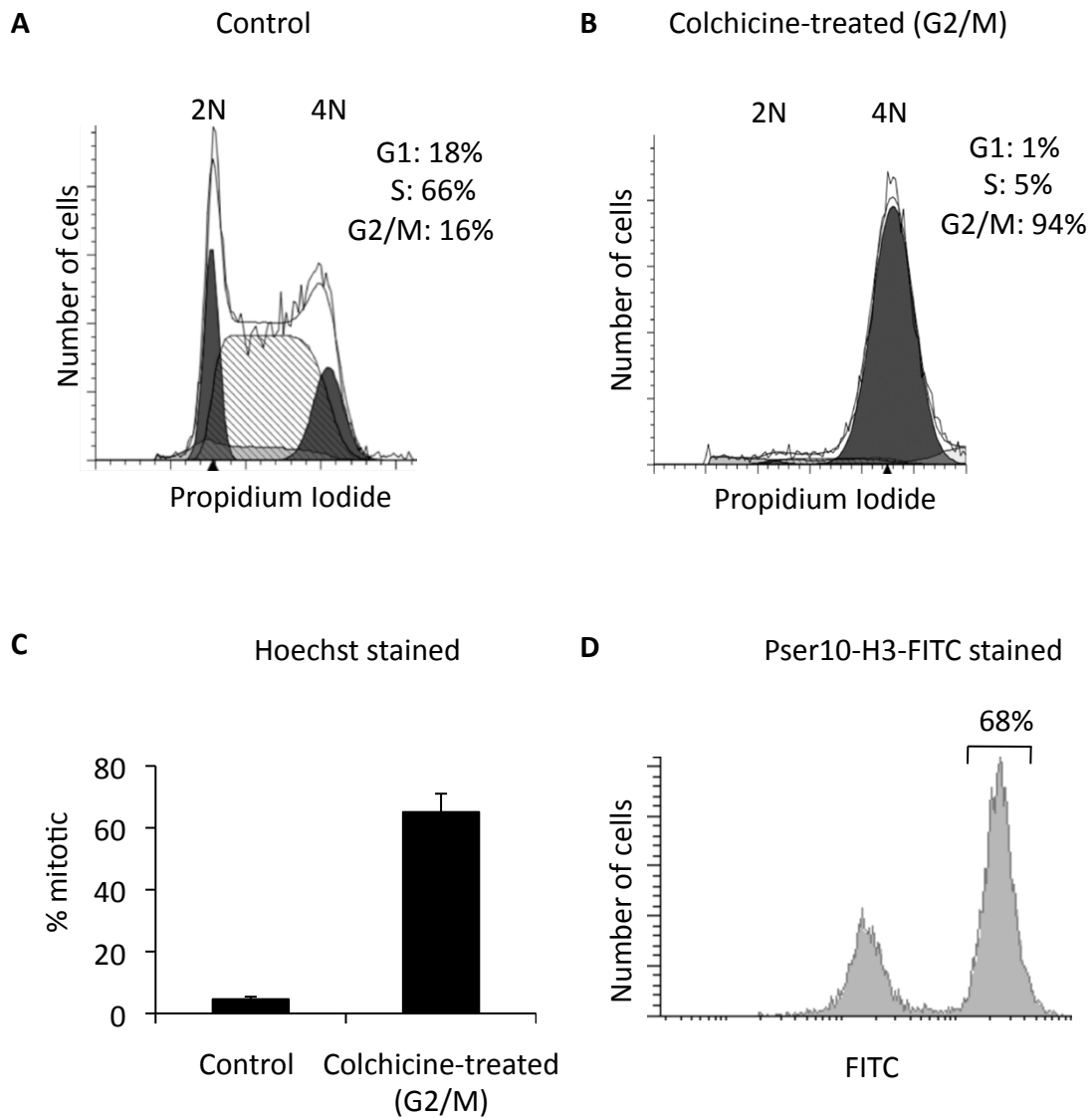
**Figure 2-1. The PcG proteins PSC, PC and dR are not excluded from mitotic chromosomes.** (A-D) Representative immunofluorescence images of *Drosophila* S2 cells stained with antibodies against dR (A), PC (B), PSC (C), or no 1° antibody (D). Left panels show Hoechst-stained DNA, and right panels immunofluorescence. Top rows are interphase cells and bottom rows are mitotic cells. (E-H) Panels are the same as A-D except that cells were extracted with detergent prior to fixation. Scale bar is 5 μm. (I) Quantification of PcG signal overlapping with DNA/PcG signal in the cytoplasm. All error bars show mean  $\pm$  s.d. in this and all other figures. \*  $P < 0.001$  (two-tailed Student's *t*-test). (J) Same as I for detergent-extracted cells.



by Western blot analysis, was carried out. This technique does not depend on cell fixation or antigen accessibility. To obtain the large amounts of mitotic cells needed for biochemical analysis, *Drosophila* S2 cells were treated with colchicine, a drug that blocks microtubule polymerization leading to metaphase arrest. At least 95% of colchicine treated cells have 4N DNA content, and about 66% of these are mitotic (Figure 2-2A-D). Colchicine-treated (hereafter referred to as G2/M) and asynchronously growing (hereafter referred to as control) cells were fractionated according to the scheme in Figure 2-3A, based on (Mendez and Stillman, 2000). The distribution of several PcG proteins across the fractions was determined by Western blotting, and the percent of each protein in each fraction was quantified. For each set of G2/M cells, the mitotic index was measured as in Figure 2-2C and distributions of proteins were corrected to account for non-mitotic cells.

To validate the fractionation procedure, the distribution of  $\beta$ -tubulin and histone H3 were determined.  $\beta$ -tubulin is found primarily in the cytosolic fraction (S2), while H3 is found primarily in the chromatin pellet (P3), as expected (Figure 2-3B, C). In mitosis, the nuclear envelope is partially broken down which may allow mixing between nuclear and cytosolic proteins. Thus, the exact nature of S2 (cytosolic), and S3 (soluble nuclear) fractions in G2/M cells is unclear, although it is expected that the S3 fraction will contain proteins that are loosely associated with chromatin in both cases.

The distribution of 8 proteins, representing several PcG complexes, were tested: PSC, PC, PH and dR (PRC1 and dRAF); E(Z) and SU(Z)12 (PRC2); Pho (PhoRC);



**Figure 2-2. Colchicine-treated *Drosophila* S2 cells are mitotically-arrested. (A-B)** Representative FACS profiles of propidium iodide stained cells, showing DNA content. Results of cell cycle analysis are shown in the upper right. Profile for control culture (**A**) and colchicine-treated (G2/M) culture (**B**). (**C**) Mitotic index of colchicine-treated cells determined by counting Hoechst-stained cells with condensed chromosomes. (**D**) Representative FACS profile of G2/M cells stained with FITC-conjugated  $\alpha$ -H3S10p. Quantification of percentage FITC-positive is indicated.

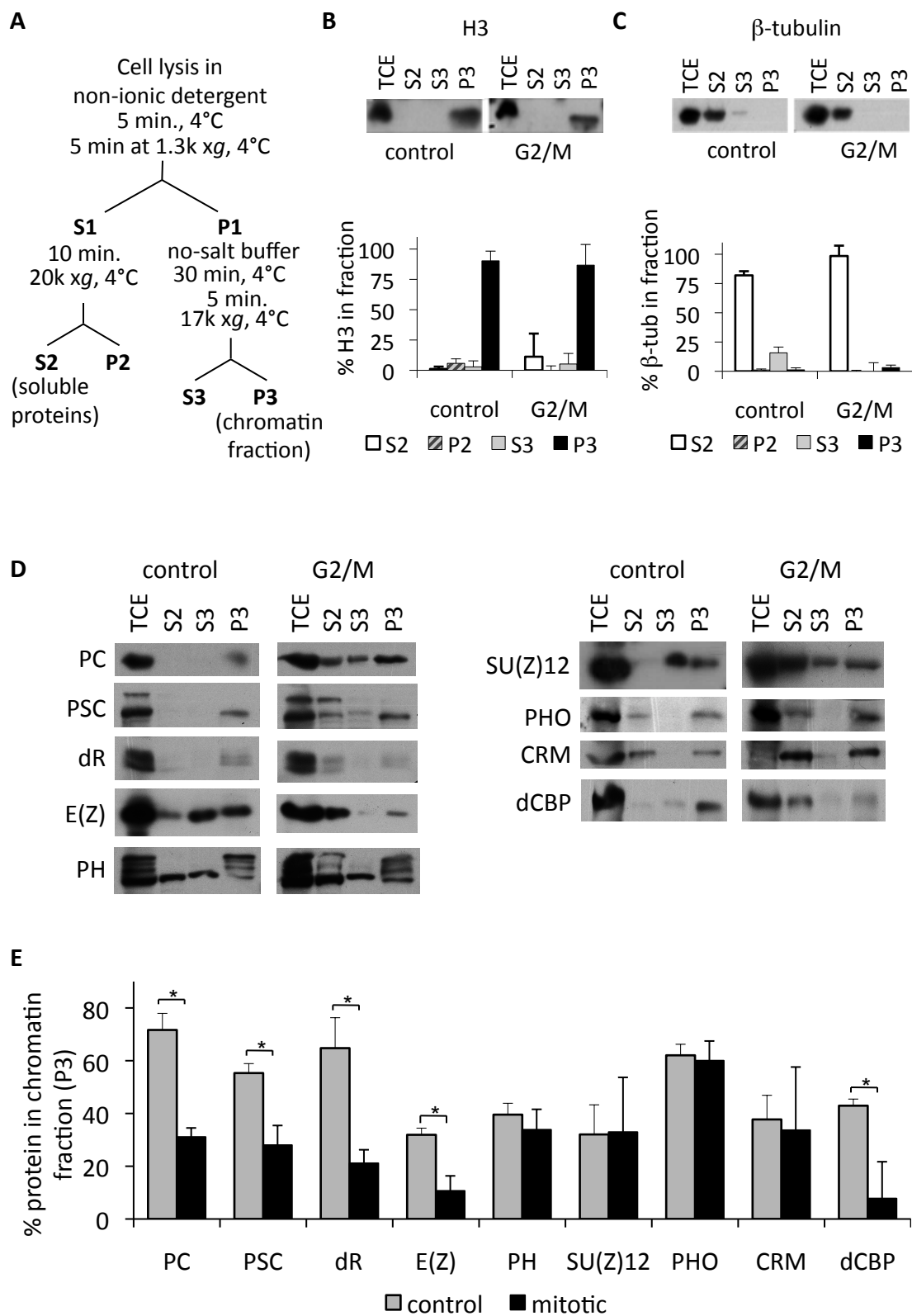
**Figure 2-3. PcG proteins fractionate with mitotic chromosomes.** (A) Schematic diagram of fractionation protocol used, adapted from Mendez and Stillman, 2000. (B, C) Representative western blot (top panels) of fractions for histone H3 (B) or  $\beta$ -tubulin (C). Quantification of the distribution of the protein in each fraction (bottom panels). G2/M samples were corrected for % of non-mitotic cells in the population according to:

$$\% P3_{\text{mitotic}} = [\%P3 - (\% \text{non-mitotic}) * \%P3_{\text{control}}] / (\% \text{mitotic})$$

(D) Representative western blots of fractions for PcG proteins and dCBP. (E) Quantification of the amount of protein in the chromatin fraction, P3, for PcG proteins and dCBP. dCBP was used as a positive control for a protein that is released in mitosis. n.d.=not determined. TCE=total cell extract.

\* $P < 0.02$  (two-tailed Student's  $t$ -test).

**Figure 2-3 (Continued)**



and Cramped (CRM). A large fraction of each PcG protein is in the chromatin pellet (P3) and soluble nuclear fraction (S3) in control cells, (Figure 2-3D, E). In G2/M cells, a portion of each PcG protein fractionates with the chromatin, even after accounting for non-mitotic cells (Figure 2-3D, E, Table 2-1). For one class of proteins (PC, PSC, dR and E(Z)), less protein is in the chromatin fraction in mitotic cells than control cells (33-51% of control). The decrease in these proteins in the chromatin fraction is reflected by an increase in the cytosolic (S2) fraction and is consistent with release of some protein from chromatin into the cytosol during mitosis. For the remaining proteins (PH, SU(Z)12, PHO and CRM) the fraction of protein in the chromatin pellet is nearly unchanged between control and mitotic cells (>85% of control).

As a positive control, the distribution of dCBP, a protein whose mammalian homolog has been reported to dissociate from mitotic chromosomes (Kouskouti and Talianidis, 2005) was analyzed (Figure 2-3D, E, Table 2-1). In contrast to the results with PcG proteins, the fraction of dCBP in the chromatin pellet (P3) in mitotic cells is only 18% of that in control cells. Thus a portion of most PcG proteins fractionates with chromatin in mitotic cells, but this fraction is decreased for some proteins. The biochemical fractionation data are consistent with the immunofluorescence data which also show decreased PC, PSC, and dR associated with chromatin (Figure 2-1).

**Table 2-1. Summary of fractionation results for PcG proteins.** Percent protein in each fraction is given  $\pm$  s.d. G2/M samples were corrected for % of non-mitotic cells in the population as described in Figure 2-3.

		S2	P2	S3	P3
PC	control	6.0 $\pm$ 1.9	0.8 $\pm$ 0.8	21.6 $\pm$ 5.6	71.7 $\pm$ 6.3
	G2/M	45.4 $\pm$ 5.1	2.3 $\pm$ 1.7	21.1 $\pm$ 0.6	31.1 $\pm$ 3.4
PSC	control	25.2 $\pm$ 2.0	1.6 $\pm$ 0.4	17.9 $\pm$ 3.8	55.3 $\pm$ 3.6
	G2/M	59.0 $\pm$ 10.9	4.1 $\pm$ 1.0	8.9 $\pm$ 6.6	28.0 $\pm$ 7.4
dR	control	16.8 $\pm$ 6.3	1.7 $\pm$ 1.8	16.7 $\pm$ 7.6	64.8 $\pm$ 11.5
	G2/M	60.9 $\pm$ 6.8	4.4 $\pm$ 3.8	13.6 $\pm$ 6.1	21.1 $\pm$ 5.1
E(Z)	control	35.0 $\pm$ 4.9	3.6 $\pm$ 5.6	29.5 $\pm$ 5.7	31.9 $\pm$ 2.5
	G2/M	84.1 $\pm$ 3.2	0.0 $\pm$ 6.1	6.3 $\pm$ 4.3	10.7 $\pm$ 5.7
PH	control	30.2 $\pm$ 2.3	3.1 $\pm$ 1.2	27.1 $\pm$ 2.5	39.6 $\pm$ 4.3
	G2/M	49.3 $\pm$ 2.0	0.0 $\pm$ 3.9	16.8 $\pm$ 3.2	33.8 $\pm$ 7.7
SU(Z)12	control	29.8 $\pm$ 9.0	1.7 $\pm$ 1.3	36.5 $\pm$ 7.4	32.0 $\pm$ 11.2
	G2/M	49.8 $\pm$ 23.2	6.9 $\pm$ 4.0	10.3 $\pm$ 6.9	32.9 $\pm$ 20.7
PHO	control	25.0 $\pm$ 4.8	7.5 $\pm$ 2.8	5.4 $\pm$ 4.8	62.0 $\pm$ 4.2
	G2/M	38.2 $\pm$ 16.3	0.0 $\pm$ 1.4	2.0 $\pm$ 7.6	60 $\pm$ 7.4
CRM	control	60.3 $\pm$ 8.9	0.6 $\pm$ 1.0	1.4 $\pm$ 0.6	37.7 $\pm$ 9.1
	G2/M	64.7 $\pm$ 25.9	0.3 $\pm$ 0.7	1.3 $\pm$ 2.3	33.7 $\pm$ 23.9
dCBP	control	21.2 $\pm$ 1.4	3.8 $\pm$ 3.5	32.0 $\pm$ 3.2	42.9 $\pm$ 2.5
	G2/M	65.1 $\pm$ 5.4	12.7 $\pm$ 6.3	14.5 $\pm$ 5.4	7.8 $\pm$ 13.9

## Discussion

How chromatin states can be propagated through mitosis is a major question in epigenetics. How PcG-dependent repression might be propagated through mitosis was investigated by systematically characterizing the behavior of PcG proteins during mitosis in *Drosophila* S2 cells. Using two different methods (immunofluorescence, biochemical fractionation) it is shown here that PcG proteins are associated with mitotic chromosomes. These results are discussed below in light of previous data and how they affect models for propagation through mitosis.

By immunofluorescence PSC, PC, and dR are associated with mitotic chromosomes, albeit at reduced levels compared with interphase cells (Figure 2-1). Previous immunofluorescence studies in *Drosophila* have shown either that PcG proteins are released from mitotic chromosomes or remain associated (Beck et al., 2010; Buchenau et al., 1998; Dietzel et al., 1999; Fanti et al., 2008; Martin and Adler, 1993; Messmer et al., 1992). For both PSC and PC, initial work demonstrated staining of mitotic chromosomes, in embryos for PSC (Martin and Adler, 1993) and in S2 cells for PC (Messmer et al., 1992), and later work localized both PSC and PC to mitotic chromosomes in larval neuroblasts (Fanti et al., 2008). One study characterized the distribution of PH, PSC, and PC carefully through mitosis in embryonic cells, and concluded that all three proteins are largely dissociated from mitotic chromosomes (Buchenau et al., 1998). Comparison of the images of PSC and PC staining of mitotic S2 cells generated in this study with those of Buchenau *et al.* (1998) from *Drosophila* embryos indicates that they are quite similar, although

Buchenau *et al.* observe clear enrichment of PSC on anaphase chromosomes relative to prophase or metaphase which was not apparent in the experiments presented here. Another study determined the subcellular localization of a PC-GFP fusion protein in embryos and larval neuroblasts and concluded that PC is largely lost from mitotic chromosomes (Dietzel et al., 1999). Examination of the images of PC in larval neuroblasts, and embryos to some extent, reveals that they are also similar to the images presented here. Both the real differences in staining patterns and the perceived differences reflected in the conflicting conclusions drawn about the localization of PcG proteins on mitotic chromosomes may depend upon details in how material was prepared and quantified.

An independent method, biochemical fractionation, was used to confirm that PcG proteins are associated with mitotic chromosomes. A fraction of all proteins tested fractionated with mitotic chromosomes, consistent with at least partial retention of each protein on mitotic chromosomes. There was little difference in the percentage of total protein that fractionates with the chromatin pellet in control vs. mitotic cells for PH, SU(Z)12, PHO and CRM, while there was a smaller percentage of total protein that fractionates with the chromatin pellet in control vs. mitotic cells for PC, PSC, dR and E(Z). These results are consistent with a model in which PcG proteins remain bound to mitotic chromosomes and may constitute the transcriptional memory of repression themselves.

The retention of PHO on mitotic chromosomes would be particularly significant when combined with the loss of other PcG proteins from mitotic



chromosomes, as PHO is thought to play a role in recruiting other PcG proteins to target sites. PHO has been shown to interact with PRC1 Core Complex (Mohd-Sarip et al., 2005; Mohd-Sarip et al., 2006), PRC1 components PC and PH (Mohd-Sarip et al., 2002; Poux et al., 2001), and PRC2 components E(Z) and ESC (Poux et al., 2001; Wang et al., 2004b). In addition the requirement for PHO and PHOL for binding of other PcG proteins to specific PREs has been demonstrated (Klymenko et al., 2006; Savla et al., 2008; Wang et al., 2004b). Thus, at sites where PHO serves to target other PcG complexes normally during development, it may serve the same function upon mitotic exit at sites that lose other PcG proteins.

The data presented here are consistent with partial release of the PcG proteins PSC, PC, dR and E(Z) during mitosis. The mechanism responsible for their release may take one of several forms. Changes to chromatin that take place during mitosis may reduce the affinity of PcG proteins for the mitotic chromatin, or conversely alterations of the PcG proteins themselves may lead to reduction of binding. Alternatively, the increase in volume associated with partial dissolution of the nuclear envelope and corresponding reduction in PcG protein concentration may lead to the dissociation of PcG protein from chromatin without change in either protein or chromatin.

Chromatin binding of both mammalian homologs of PSC is regulated by phosphorylation. Hypophosphorylated MEL-18 is lost from mitotic chromatin, while BMI-1 is phosphorylated during mitosis and this phosphorylated form is released from chromatin (Elderkin et al., 2007; Voncken et al., 1999). Comparison of PSC in

the fractions from control cells with those from G2/M cells shows the appearance of a more slowly migrating band in G2/M cells that is largely found in the cytosolic fraction but not in the chromatin fraction (Figure 2-3D). It is possible that this band represents a phosphorylated form of PSC that is released from chromatin.

Treatment of the cytosolic fraction with phosphatase could test this hypothesis, while phosphorylation and *in vitro* binding experiments with recombinant PSC could be used to determine if a phosphorylated form of PSC exhibits reduced chromatin binding.

It has been shown that one of the mammalian homologs of E(Z), EZH2 is phosphorylated by cyclin-dependent kinase 1 and 2 (CDK1 and CDK2, respectively), and that the phosphorylated form is enriched in mitosis. The phosphorylated form was shown to be important for gene silencing, binding of EZH2 to ncRNAs and for recruitment of EZH2 to target promoters (Chen et al., 2010; Kaneko et al., 2010). Thus, it is possible that binding of the phosphorylated form of EZH2 to ncRNAs during mitosis may serve as a mechanism for maintenance of EZH2 through mitosis. There is no evidence for phosphorylation of E(Z) in the experiments performed here, although it is possible that the antibody used does not recognize a phosphorylated form of the protein, the phosphorylated form does not exhibit a mobility shift, or that the phosphorylated form exists as a small percentage of the total population. In fact Kaneko *et al.* (2010) do not observe mobility shift of the phosphorylated forms of EZH2 and estimate that the phosphorylated forms represent only ~1-3% of total EZH2 in asynchronously-growing cells, which would be ~3-9% in G2/M cells.

Even if E(Z) is phosphorylated in mitosis, the role for ncRNA in targeting PcG proteins to chromatin in *Drosophila* is not yet clear (see Introduction).

It is possible a change in protein binding partners may trigger the release of PC, PSC, dR and E(Z) from mitotic chromatin. The partial dissociation of the nuclear envelope may allow for interactions that were previously prevented by subcellular compartmentalization. The fact that chromatin binding by PC is reduced during mitosis while the fractionation of fellow PRC1 component PH remains unchanged and E(Z) is reduced while fellow PRC2 component SU(Z)12 remains unchanged may indicate partial dissociation of PRC1 and PRC2 during mitosis, which may or may not be accompanied by new interactions. Data from our laboratory suggests mitotic-specific complexes do exist (A. Wani, personal communication). Retention of a partial complex on mitotic chromatin may facilitate the reformation of complete complexes upon mitotic exit.

It has been shown in contexts other than mitosis that phosphorylation of H3Ser28 correlates with a decrease in chromatin binding by BMI1 and EZH2 (Lau and Cheung, 2011). In a separate study it was shown that the H3K27me3S28p double modification causes abolition of H3-tail peptide binding by the mammalian PRC2 components EZH2, SUZ12 and EED and reduction of binding by the PC homolog CBX8, in addition loss of these proteins from gene promoters (Gehani et al., 2010). As the H3Ser28p modification also occurs during mitosis it may be a mechanism for displacement of PcG proteins from mitotic chromatin. Partial dissociation of E(Z) and PC but not SU(Z)12 is observed here. Similarly it has been demonstrated that

the chromodomain-containing protein HP1 that recognizes H3K9me3 dissociates from mitotic chromatin due to phosphorylation of the nearby residue H3S10 (Fischle et al., 2005; Hirota et al., 2005). As the PC protein also contains a chromodomain that binds a residue (H3K27me3) neighboring a mitosis-specific phosphorylation site (H3S28), PC may be released from mitotic chromatin in an analogous fashion. H3 tail peptide binding experiments with PC and singly and doubly modified peptides (H3K27me3 and H3K27me3S28p), and use of Aurora-B inhibitors or knockdown coupled with PC localization during mitosis could uncover whether H3S28p is responsible for PC dissociation from mitotic chromosomes.

While the results described here are consistent with a model in which PcG proteins remain bound to target sites and contribute to the transcriptional memory themselves, it has not been demonstrated that these proteins actually remain bound to target sites in mitosis. Previous studies indicate that some chromosomal proteins remain chromatin associated during mitosis but change their distribution during mitosis (Blobel et al., 2009; Platero et al., 1998). Some proteins also associate non-specifically with mitotic chromosomes, traveling as passengers to ensure equal segregation to both daughter cells (reviewed in Delcuve et al., 2008; Zaidi et al., 2011). It is possible that the PcG protein that fractionates with chromatin and is seen on mitotic chromosomes by immunofluorescence is not specifically bound to target sites, or the proteins may be binding to new sites. Loss of chromatin-associating protein during mitosis (for PSC, PC, dR and E(Z)) could be explained by partial loss of the protein from all interphase sites or preferential retention at some

sites and complete loss at others. The question of whether PcG proteins remain bound to target sites is the subject of the next chapter.

## **Experimental Procedures**

### *Cell culture and synchronization*

*Drosophila* S2 cells (Invitrogen, Carlsbad, CA) were cultured in ESF 921 media (Expression Systems, Woodland, CA) at a density between 1 and  $7 \times 10^6$  cells/mL in shaking flasks at 27°C. For synchronization, colchicine (Sigma) was added to cells to a final concentration of 350ng/ml (880nM). Cells were harvested 15 hours later by centrifugation at 400 x *g* for 5 min. at 4°C and then centrifuged twice (480 x *g* for 5 min.) through a 20% sucrose cushion to remove cell debris. Aliquots of cells were collected, stained with propidium iodide, and analyzed for DNA content on an LSRII cell sorter (BD Biosciences). Mitotic index was determined by staining an aliquot of cells with Hoescht and visually scoring cells with condensed chromosomes as mitotic. Similar results were obtained by staining cells with anti-H3Ser10p and carrying out FACS analysis.

### *Antibodies*

The affinity-purified anti-PSC antibody raised against PSC aa 521-869 was previously described (Francis et al., 2009). Antibodies against PC, PH, PHO and SU(Z)12 were kind gifts from J. Mueller. The antibody against dRING was a gift from R. Jones. The anti-CRM antibody was a gift from Walter Gehring, and the anti-dCBP

antibody was a kind gift from Alexander Mazo. The anti-E(Z) antibody, (dL-19), and the anti- $\beta$ -tubulin antibody, (d-140) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-H3 antibody, ab39655, was purchased from Abcam (Cambridge, MA). The antibody to H3Ser10p was purchased from Millipore (Billerica, MA).

### *Immunofluorescence and Imaging*

Immunofluorescence was performed as previously described (Fanti et al., 2008).  $1 \times 10^6$  *Drosophila* S2 cells were plated on concanavalin A (0.5mg/mL) coated coverslips in 6-well plates and allowed to attach overnight. For detergent-extraction, cells were first incubated in 1% digitonin, 20mM HEPES, pH 7.3, 110mM KOAc, 5mM NaOAc, 2mM MgOAc, 1mM EGTA for 5 min. on ice. The rest of the staining procedure was the same for detergent-extracted and unextracted cells: Cells were washed at room temperature 2x with 0.7% NaCl, incubated for 10min. in 0.5% sodium citrate, and fixed for 8min. in 50% methanol, 20% acetic acid. Cells were washed 5min. in PBS and permeabilized for 10min. in 1x PBS + 1% triton-X 100. Cells were blocked for 30min. at room temperature in 5% milk in 1x PBS, rinsed in 1x PBS and incubated with primary antibody diluted 1:200 in 1% BSA + 1x PBS overnight at 4°C. Cells were then washed 3x 5 min. in 1x PBS and incubated 2 hrs. at room temperature in 1:200 dilution of secondary antibody in 1% BSA in 1x PBS + 0.1% Triton-X 100. Cells were washed 2x 5 min. in 1x PBS and stained 10 min. with Hoechst (0.5mg/mL), washed 5min. in 1x PBS and mounted on slides.

Cells were visualized on a Zeiss LSM700 inverted confocal microscope. 0.7 $\mu$ m optical sections were taken using a 63x objective. Laser power and gain were kept constant for all images taken from the same slide. Images were quantified for each antibody using ImageJ. For each cell, a DNA mask was chosen by applying a threshold to the DNA channel using the Li method and selecting the outline of the DNA at the signal/background border. Average signal intensity for the PcG channel within this DNA mask was recorded to give PcG<sub>DNA</sub>, the PcG signal that overlaps DNA. A cell mask was chosen by applying a threshold to the PcG channel using the Li method and selecting the outline of the cell at the signal/background border. A cytoplasmic mask was created by subtracting the DNA mask from the cell mask. Average signal intensity for the PcG channel within the cytoplasmic mask was recorded to give PcG<sub>cyto</sub>, the PcG signal in the cytosol. PcG<sub>DNA</sub>/PcG<sub>cyto</sub> were calculated and averaged for mitotic and interphase cells.

#### *Cell fractionation and chromatin isolation*

Cell fractionation was carried out as in (Méndez and Stillman, 2000) with minor changes. 3.5x10<sup>7</sup> asynchronously-growing, control, or colchicine-treated, G2/M, cells were treated with 2 units of DNaseI and incubated on ice for 1 hr. for total cell extract (TCE) or fractionated by resuspension to 7x10<sup>7</sup> cells/mL in Buffer A (10mM HEPES, pH 7.9, 10mM KCl, 1.5mM MgCl<sub>2</sub>, 0.34M sucrose, 10% glycerol) plus 0.1% Triton-X 100, 1mM DTT, 50mg/mL TLCK, 10mg/mL aprotinin, 16mg/mL benzamidine, 10mg/mL leupeptin, 2mg/mL pepstatin, 10mg/mL phenanthroline, and 0.2mM

PMSF; and incubated on ice for 5 min. The samples were centrifuged ( $1,300 \times g$ , 4 min,  $4^{\circ}\text{C}$ ) to give pellet 1 (P1) and supernatant (S1). S1 was centrifuged ( $20,000 \times g$ , 15min,  $4^{\circ}\text{C}$ ) to give supernatant (S2) and pellet (P2). P1 was washed once in Buffer A and then lysed in Buffer B (3mM EDTA, 0.2mM EGTA, 1mM DTT, protease inhibitors as described above). Insoluble chromatin was collected by centrifugation ( $1,700 \times g$ , 4 min.,  $4^{\circ}\text{C}$ ) to give pellet (P3) and supernatant (S3). P3 was washed once in Buffer B, centrifuged again under the same conditions and resuspended in SDS loading buffer and sonicated for 5 sec. with a VibraCell sonicator (Sonics & Materials, Inc., Newtown, CT) using a microtip at 25% amplitude. Fractions were run on 8% or 15% SDS PAGE gels, transferred to nitrocellulose, blotted and developed using HRP. Blots were scanned on a Typhoon Imager and quantified with ImageQuant.



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## **Chapter 3**

**PcG proteins are retained at specific sites during mitosis**

## Introduction

The fate of factors involved in regulation of transcription during mitosis may have implications for the re-establishment of gene expression states upon mitotic exit, or for their reprogramming (reviewed in Egli et al., 2008). While many sequence-specific transcription factors dissociate from mitotic chromatin, the existence of several molecular features have been posited to serve as molecular 'bookmarks' to mark certain genes for transcriptional reactivation. Such bookmarks include both general and sequence specific transcription factors, histone modifications and non-histone chromatin proteins (reviewed in Sarge and Park-Sarge, 2009).

Chromatin immunoprecipitation (ChIP) performed on cells in mitosis has demonstrated the retention of certain proteins at target sites. For example Runx2 and BRD4 bind to target sites in mitosis and this binding has been found to be important for target gene expression in G1 (Mochizuki et al., 2008; Young et al., 2007). Both ChIP-chip and ChIP-SEQ performed on mitotic chromosomes has uncovered novel roles for the proteins studied in mitotic bookmarking that would not have been discovered through ChIP-qPCR alone. The distribution of target sites of the protein MLL was shown to change during mitosis. MLL bound to a new subset of sites exclusively during mitosis, and it was found that MLL was required for the rapid reactivation of these sites during the subsequent G1 (Blobel et al., 2009). Likewise ChIP-SEQ of the histone variant H2A.Z, a histone variant often found within the +1 nucleosome at transcription start sites (TSSs) of active genes, revealed that



nucleosomes containing H2A.Z also relocate during mitosis—they shift upstream to cover the TSS during mitosis. It was suggested that this may serve as both a mechanism of transcriptional repression during mitosis and a bookmark for reactivation upon mitotic exit (Kelly et al., 2010).

Polycomb Group proteins mediate stable repression of target genes through epigenetic mechanisms and assemble into multi-protein complexes with an array of effects on chromatin (reviewed in Müller and Verrijzer, 2009; Pirrotta, 1998; Ringrose and Paro, 2004; Simon and Tamkun, 2002). It is unknown how PcG-dependent repression can be inherited through mitosis, although some studies in mammalian cells have identified mechanisms that may contribute to maintaining association of PRC2 with chromatin. The E(Z) homolog EZH2 is phosphorylated during mitosis, which enhances its binding to ncRNAs which can target the protein to chromatin (Kaneko et al., 2010). It has also been found that PRC2 can recognize the H3K27me3 modification that it creates, and that binding to the methylated histone tail stimulates PRC2 methyltransferase activity. This suggests a feedback loop for maintenance of PRC2 binding and H3K27me3. EZH2 has also been observed on mitotic chromosomes by immunofluorescence, although it is not known if it remains associated with its target genes (Hansen et al., 2008; Xu et al., 2010).

The finding that *Drosophila* PcG proteins are associated with mitotic chromosomes (Chapter 2) raises the possibility that PcG proteins function as mitotic bookmarks to maintain gene repression. PcG proteins may remain bound to target

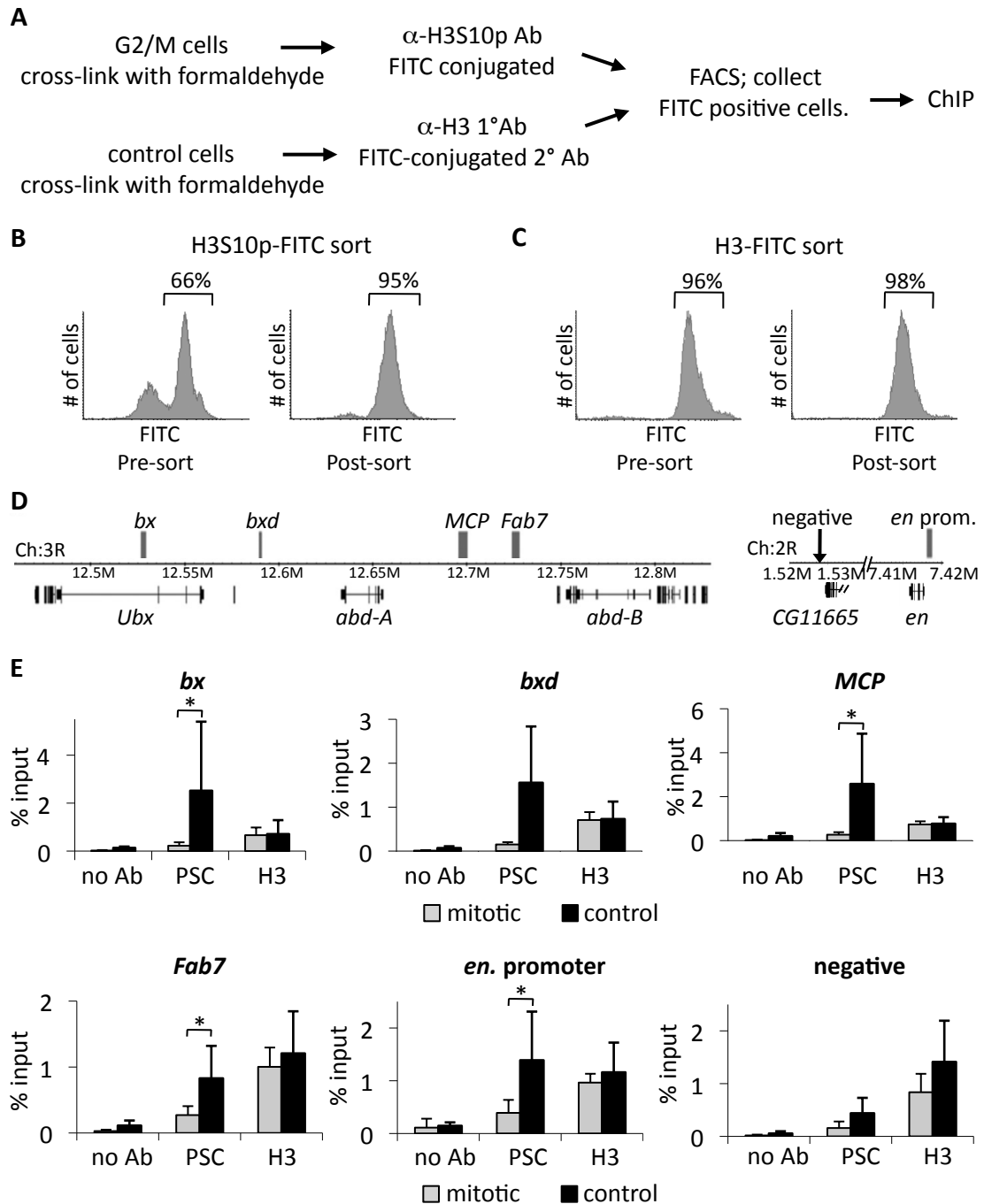
genes in mitosis, like Runx2 or BRD4, or shift to new sites in mitosis, like MLL or H2A.Z.

To test whether PcG proteins are mitotic bookmarks, PcG protein localization to specific genes in mitotic cells was determined by Chromatin Immunoprecipitation (ChIP) followed by genome-wide sequencing (ChIP-SEQ) of mitotic cells and ChIP of G2/M cells.

## Results

*Polycomb Group proteins are not detected at Polycomb Response Elements (PREs) in pure populations of mitotic cells.*

Our fractionation and immunofluorescence analysis indicates that PcG proteins are associated with chromatin in mitotic cells (Chapter 2). To determine whether PcG proteins remain localized to their specific target sites during mitosis chromatin immunoprecipitation (ChIP) followed by genome-wide sequencing (ChIP-SEQ) on mitotic cells was carried out. Pure populations of mitotic cells were isolated from colchicine-treated cultures using Fluorescence Activated Cell Sorting (FACS) with antibodies to histone H3 phosphorylated at serine 10 (H3S10p), which is a reliable marker of mitotic cells (Figure 3-1A). A detailed description of this method of isolating pure populations of mitotic cells is presented in Chapter 4 (Follmer and Francis, 2012). Starting with a G2/M population that was ~66% mitotic, we obtained H3S10p-sorted cells that are ≥95% mitotic (Figure 3-1B). To control for the FACS procedure, we sorted control (untreated) cells with antibodies to histone H3.



**Figure 3-1. PSC binding is very low at PREs in mitotic cells.** (A) Schematic diagram of the FACS sorting protocol to isolate mitotic cells based on H3S10p immunoreactivity. Control cultures were sorted with antibodies to histone H3. (B-C) Representative FACS profiles of G2/M cells stained with FITC-conjugated α-H3S10p (B) or α-H3 and a FITC-conjugated 2° antibody (C) before (left) and after (right) sorting. Quantification of percentage FITC-positive is indicated. (D) Schematic diagram of part of the BX-C and the *engrailed* locus. (||) indicates PREs. (E) ChIP-qPCR for PSC and H3 in H3-sorted and H3S10p-sorted cells. \* $P < 0.05$  (two-tailed Student's *t*-test comparing mitotic and control).

At least 95% of control H3-sorted cells were H3-FITC positive in the post-sorting analysis (Figure 3-1C). We used biotinylated antibodies to PSC and streptavidin-coated beads for ChIP-qPCR to avoid isolation of the antibodies used for sorting (and associated chromatin). To analyze the distribution of PH, we used a stable S2 cell line expressing low levels of biotinylated PH instead of antibodies.

PSC localization was analyzed at several PcG binding sites within the well-characterized Bithorax Complex (BX-C) of *Hox* genes: *bx*, *bxl*, *Fab-7* and *MCP* PREs; at a site within the *engrailed (en)* locus and at a negative site (Figure 3-1C, D). PSC localizes to each PRE and the site within the *en* locus but not to the negative site in control, H3-sorted cells. In H3S10p-sorted, mitotic cells, however levels of PSC at the PREs and at the *en* site are indistinguishable from the level at the negative site (Figure 3-1E). In contrast, histone H3 was present at similar levels in mitotic and control cells (Figure 3-1E). PH behaved similarly to PSC in a smaller number of experiments (not shown). We conclude that PSC is not detected at PREs in the BX-C and *en* locus in mitotic cells.

#### *Genome-wide binding profiles of PSC and PH reveal reduced chromatin binding in mitosis.*

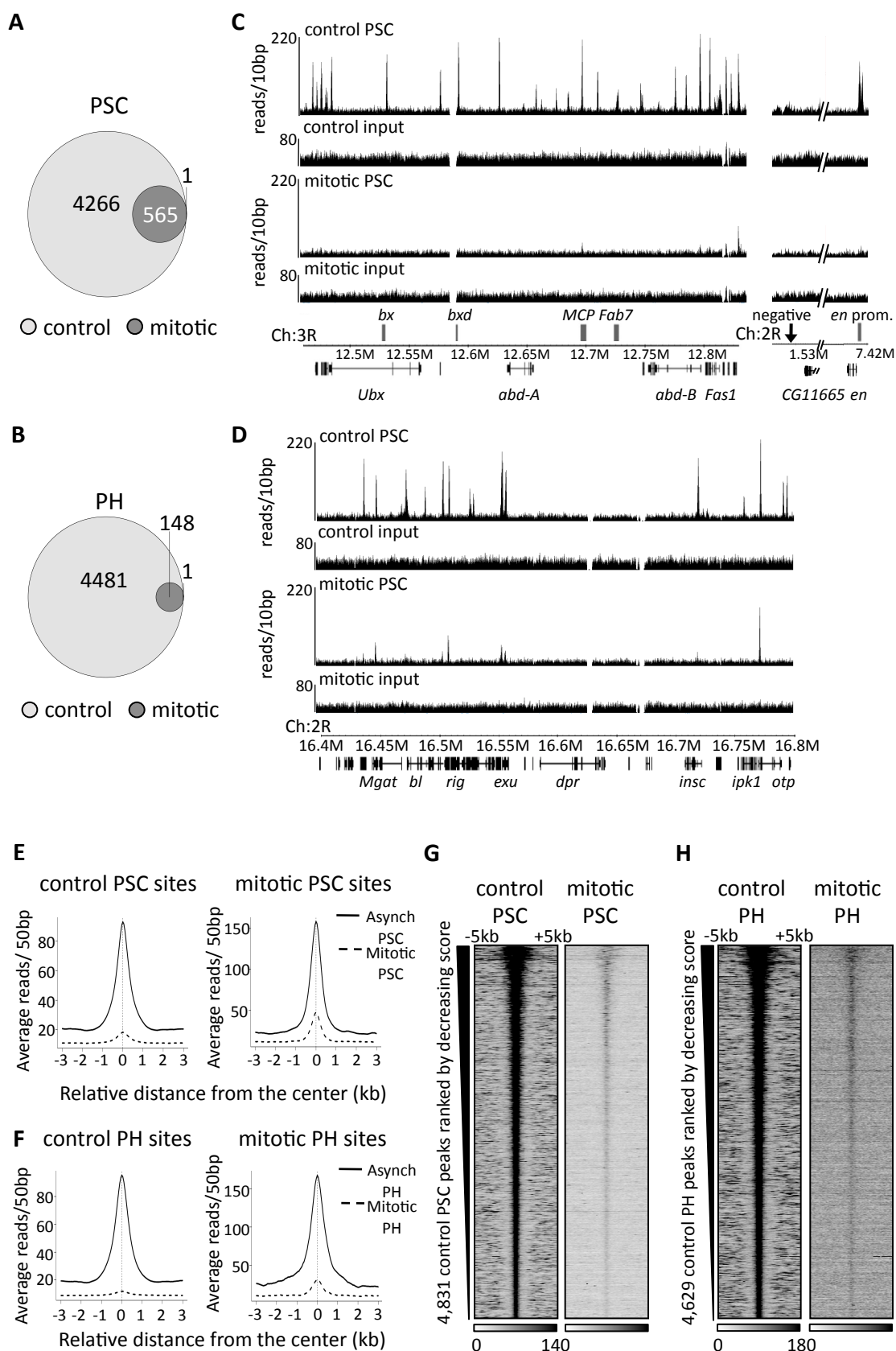
To determine if PSC and PH are bound to any specific sites on mitotic chromosomes, ChIP followed by high throughput sequencing (ChIP-SEQ) was carried out. Immunoprecipitated and input DNA from FACS-sorted mitotic and control cells was sequenced to generate genome-wide binding profiles for both PSC and PH.

Between 4.9-16.3 million reads were uniquely mapped to the genome for each sample. 4,831 and 4,629 binding sites in control cells were identified for PSC and PH, respectively, using the MACS algorithm at a 5% false discovery rate (FDR) (Zhang, 2008) (Figure 3-2A, B). Two biological replicates of PSC binding profiles from control cells are in good agreement (Pearson's correlation coefficient,  $r = 0.97$ ). The PSC and PH binding profiles are nearly identical, indicating a very high degree of colocalization for the two proteins, in agreement with other studies ( $r = 0.96$  (control);  $r = 0.97$  (mitotic)). Only one ChIP-SEQ experiment was carried out with PH but was included in the analysis because of the high overlap with the two biological replicates with PSC. Comparing our data with PSC to other published data sets, PSC overlaps with 69% (Enderle et al., 2011) and 24% (Kharchenko et al., 2010; modEncode, 2010) of peaks from S2 cells, 27% of peaks in BG3 cells, and 56% of peaks from Kc cells (Kharchenko et al., 2010; modEncode, 2010).

The same parameters were used to call peaks from data from mitotic cells and many peaks were identified in profiles for both PSC and PH. For PSC, an FDR of 5% was used and 566 peaks were identified. For PH, the signal to noise in the profile from mitotic cells was lower than that for PSC; therefore a less stringent cutoff for peaks was used, and 149 peaks were identified, 93% of which are also mitotic peaks of PSC. These peaks are a subset of the peaks from control cells; no new peaks were identified in mitotic cells (Figure 3-2A-D). Profiles averaging over all control sites for each protein confirm that sequenced reads are dramatically reduced in mitotic cells relative to control cells (Figure 3-2E, F left panels). Profiles of PSC and PH averaging

**Figure 3-2. Genome-wide analysis reveals PSC is retained at specific sites on mitotic chromosomes.** (A) Venn diagram depicting overlap of peaks of PSC binding in control (H3-sorted, asynchronous) cells and mitotic (H3S10p-sorted) cells. (B) Venn diagram depicting overlap of peaks of PH binding in control cells and mitotic cells. (C) Sequence tracks from ChIP-SEQ showing PSC binding in control S2 cells (top track) and in mitotic S2 cells (bottom track) over the BX-C and the *engrailed* locus. Y-axis is average sequence reads/10bp. Chromosome position and gene models are shown at the bottom. (D) Sequence tracks for PSC binding in control S2 cells (top track) and in mitotic S2 cells (bottom track) over a 400kbp region of chromosome 2 with several mitotic peaks. Y-axis is average sequence reads/10bp. Chromosome position and gene models are shown at the bottom. (E) Average read density in 10bp windows for PSC in control cells (solid line) and mitotic cells (dashed line) surrounding peaks of PSC in control S2 cells (left) and in mitotic cells (right). (F) Average read density in 10bp windows for PH in control cells (solid line) and mitotic cells (dashed line) surrounding peaks of PH in control S2 cells (left) and in mitotic cells (right). (G) Heatmap showing average read density in 50bp windows for PSC in control cells (left) and mitotic cells (right) surrounding ranked peaks of PSC in control S2 cells. Range is depicted in grayscale bar, below. (H) Heatmap showing average read density in 50bp windows for PH in control cells (left) and mitotic cells (right) surrounding ranked peaks of PH in control S2 cells. Range is depicted in grayscale bar, below.

**Figure 3-2 (Continued)**

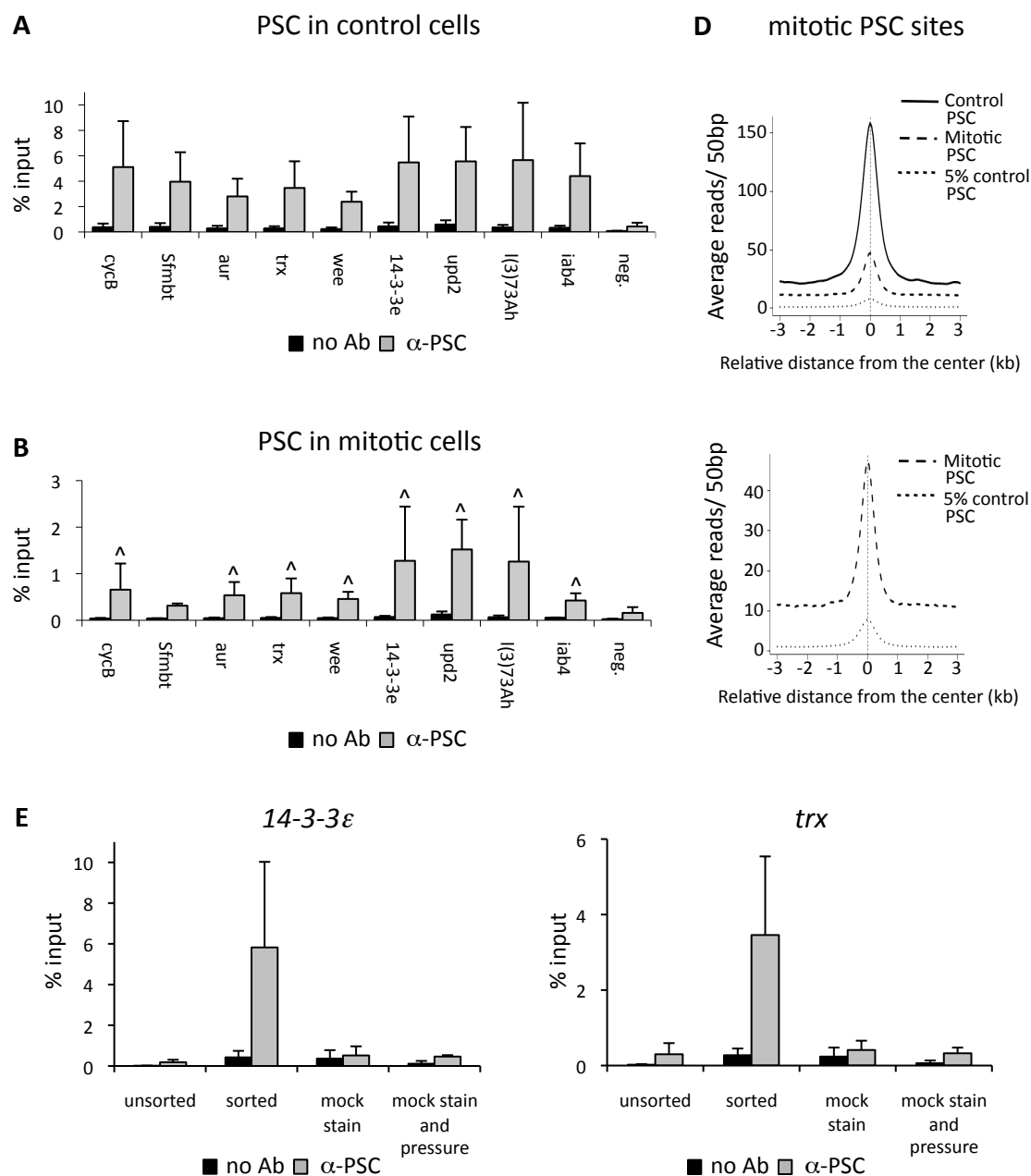


over only mitotic sites in control and mitotic cells reveal a reduction in sequence reads even at these persistent sites relative to control cells (Figure 3-2E, F right panels). Visualization of average density over all asynchronous sites as a heatmap reveals that although not enough binding persists at a majority of sites in mitotic cells to be identified as a peak, a slight enrichment remains (Figure 3-2G, H). Thus, two results are apparent: 1) both PSC and PH are lost at a majority of their binding sites in mitosis; and 2) both proteins are retained at specific sites.

Examination of PSC and PH profiles at the BX-C and *en* locus confirms the results observed by qPCR (Figures 3-2C and 3-1E), which is that both proteins are reduced in these regions in mitosis. However, other peaks throughout the genome are clearly retained (Figures 3-2D and 3-3A,B). Visual inspection of peaks reveals that some peaks are retained in mitosis while other neighboring peaks of similar size are lost. This argues that contamination of the sorted mitotic cells with non-mitotic cells (up to 5%) does not account for the mitotic peaks observed (Figure 3-2D). As a separate test of this, an average profile using 5% of the control reads was created and compared with the average profile of reads from mitotic cells (Figure 3-3C). The 5% control profile shows much less enrichment than the averaged mitotic peaks, indicating that the mitotic peaks are likely not due to contamination with non-mitotic cells.

qPCR was used to validate 9 peaks that are present in both control and mitotic cells (Figure 3-3A, B). PSC is detectable at all of these sites but that the signals are lower in mitotic than control cells, consistent with the decreased





**Figure 3-3. Validation of PSC peaks in control and mitotic cells.** (A) ChIP-qPCR for PSC in control cells at nine binding sites identified by ChIP-SEQ and a negative site. (B) ChIP-qPCR for PSC in mitotic cells at nine binding sites identified by ChIP-SEQ and a negative site. ^  $P < 0.05$ , Student's  $t$ -test, PSC at each site vs. PSC at the negative site. (C) Average read density in 10bp windows for PSC in control cells (solid line), mitotic cells (dashed line) and 5% of control cell reads (dotted line) at mitotic peak regions (top). Average read density plot showing only profiles for PSC in mitotic cells (dashed line) and 5% of control cell reads (dotted line) (bottom). (D, E) ChIP-qPCR for PSC at the *14-3-3ε* promoter (D) and the *trx* gene (E) in cells that are unsorted, sorted, mock stained or mock stained plus put under pressure.

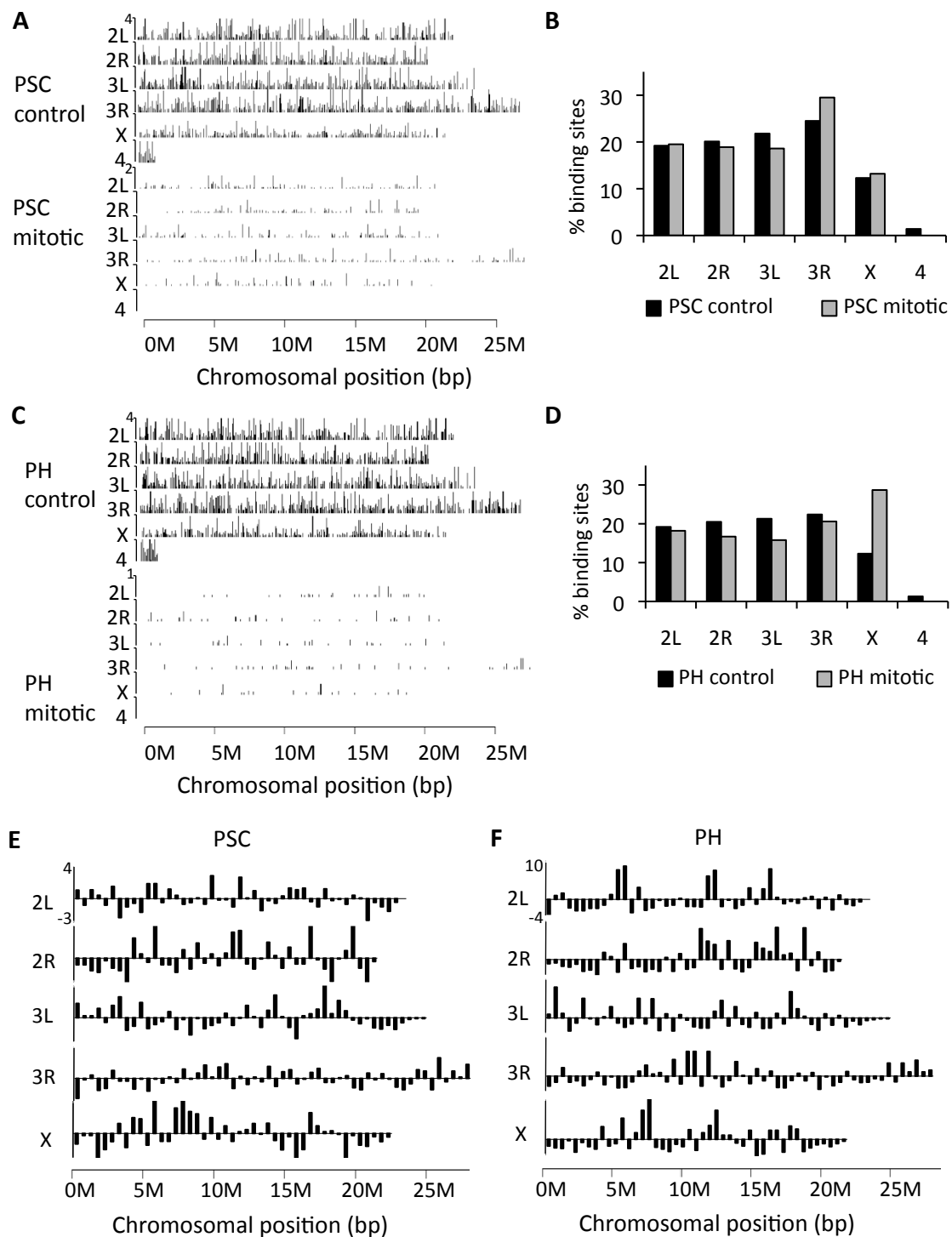
numbers of reads at mitotic sites vs. control sites (Figure 3-2E). It should be noted that while these sites are reproducibly observed in sorted cells, PSC binding at these sites is not detectable in unsorted cells. The peaks are detected in sorted cells when a biotinylated antibody (for PSC) or a biotin-tagged protein (b-PH) is used (Figure 3-2), suggesting cross-reactivity of antibodies is not the cause of the appearance of these peaks in sorted cells. ChIP performed on sorted cells without an antibody in an untagged cell line does not result in enrichment of these DNA fragments (Figure 3-3A,B), nor does ChIP performed on cells stained with either sorting antibody in absence of sorting (data not shown), which suggests neither non-specific pull down by the streptavidin beads nor interference of the sorting antibody is the cause. Thus these sites most likely represent sites of PSC binding with low accessibility to ChIP that become accessible during the staining and sorting procedure. In isolation the staining procedure without addition of the sorting antibody (mock stain), sorting without staining, and mock staining in addition to exposing cells to pressure equivalent to that used during the sorting procedure cannot recapitulate this greater accessibility (Figure 3-3D and data not shown), suggesting that the combination of staining and sorting is required to observe this greater accessibility of sites.

*PSC and PH are retained at chromatin domain borders in mitosis.*

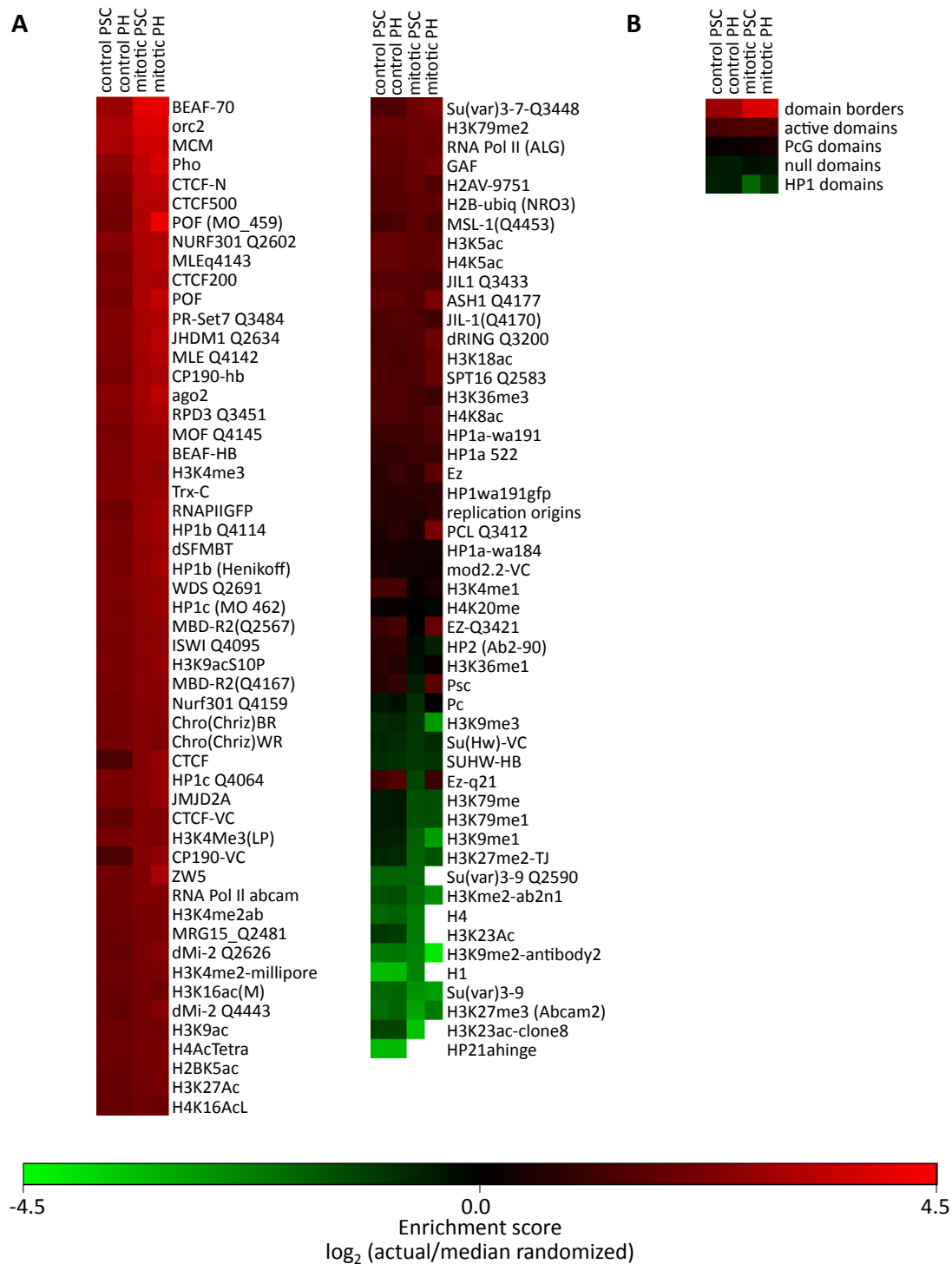
Because the mitotic binding sites for PSC and PH did not include well-known target sites the mitotic sites were analyzed in several ways to gain insight into their potential significance. Visual examination of the distribution of PSC and PH sites

over the chromosomes revealed that peaks are present on all chromosomes in control and mitotic cells, except for chromosome 4 where all mitotic peaks are lost (Figure 3-4A-C). Quantification of the percentage of total sites per chromosome confirmed that the fraction of sites per chromosome remained relatively constant among the chromosomes between the mitotic and control binding sites with the exception of chromosome 4. Analysis of the distribution of persistent sites within each chromosome arm suggests loss of sites from large regions along the chromosomes (Figure 3-4E-F) although the significance of this is not clear.

Next the binding profiles of PSC and PH from both control and mitotic cells were compared with all chromatin-bound protein profiles from *Drosophila* S2 cells published by the modEncode consortium (modEncode, 2010). Several proteins exhibited a high degree of overlap with binding profiles for PSC and PH in both control and mitotic cells including the insulator proteins CP190, BEAF, and the mitotic spindle protein Chromator (Figure 3-5). Overlap with these proteins is higher for mitotic sites than total sites, suggesting overlapping sites are preferentially retained in mitosis. The insulator protein CTCF is also enriched at mitotic sites in comparison with control sites. These four proteins were recently identified as proteins that demarcate borders between physical and functional domains that exist in the *Drosophila* genome (Sexton et al., 2012). Borders are regions identified by their paucity of long-range interactions. PSC binds 65% of domain borders in control cells, which comprises 16% of total PSC binding sites (Figure 3-6B). Interestingly, 28% of all mitotic PSC sites overlap borders, indicating that these sites are



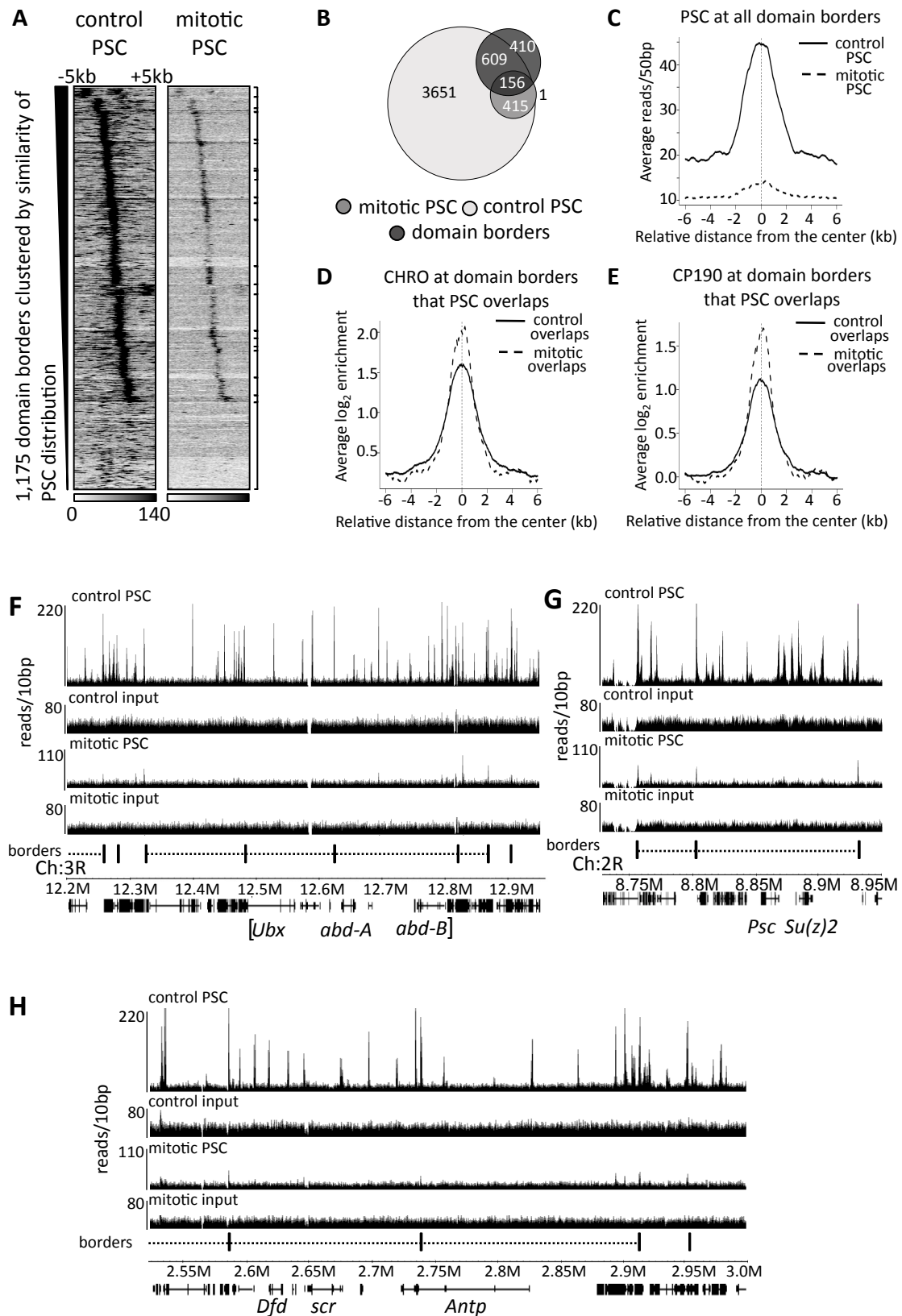
**Figure 3-4. PSC and PH distribution on chromosomes in mitosis.** (A) Distribution of PSC binding sites across chromosomes in control (top panel) and mitotic (bottom panel) cells. X-axis is chromosomal position and Y-axis is peak height given by relative sequence reads. (B) Quantification of PSC peaks per chromosome. (C, D) Same as A and B for PH. (E) Plot of difference in % of binding sites per 5Mbp window between control and mitotic distributions for PSC across chromosomes. (F) Same as E for PH.



**Figure 3-5. Overlap of PSC and PH binding profiles in control and mitotic cells with published datasets.** (A) Heatmap of enrichment of control and mitotic PSC and PH with modENCODE datasets (and ago2), sorted by decreasing enrichment by mitotic PSC. (B) Heatmap of enrichment of control and mitotic PSC and PH with domains and domain borders, sorted by decreasing enrichment by mitotic PSC. Empty boxes indicate no overlap.

**Figure 3-6. PSC and PH preferentially bind domain borders in mitosis.** (A) Heatmap showing average read density in 50bp windows for PSC in control cells (left) and mitotic cells (right) surrounding all domain borders. Borders are clustered by similarity to the PSC profiles. Range is depicted in grayscale bar below. (B) Venn diagram showing overlap between domain borders and PSC binding sites in control and mitotic cells. (C) Average profile plot of PSC in control and mitotic cells surrounding domain borders. (D-E) Average profile plot of Chromator (CHRO) (D) or CP190 (E) surrounding borders at which PSC binds in control and mitotic sites. (F) Sequence tracks from ChIP-SEQ showing PSC binding in control and mitotic cells over the BX-C and surrounding regions. Domain borders are indicated below the tracks, and are represented by (|). PcG domains identified by Sexton *et al.* (2012) are indicated by dashed lines, and the BX-C is indicated by brackets below the gene models. (G, H) Sequence tracks from the *Psc/Su(z)2* locus (G) and ANT-C (H) showing PSC binding in control and mitotic cells in relation to borders.

**Figure 3-6 (Continued)**



preferentially retained in mitosis (Figure 3-6B). Both average profiles and heatmaps in control and mitotic cells show enrichment of PSC at domain borders (Figure 3-6A, C). Chromator and CP190 are enriched at the domain borders that are bound by PSC in both mitotic and control cells (Figure 3-6 D, E). Thus, one class of persistent binding sites for PSC is at borders of chromatin domains which are marked by Chromator and CP190.

Re-examination of PSC binding at the BX-C in mitotic cells in the context of chromatin domains shows that while PSC binding is lost within the BX-C, PSC remains bound at the borders of PcG domains that encompass the cluster (Figure 3-6 F). The same pattern was observed at the ANT-C and the *Psc/Su(z)2* complex (Figure 3-6 G, H). Thus, at least some large domains of PcG protein binding are flanked by persistent peaks in mitosis. PcG binding sites within these clusters (none of which persist in mitosis) engage in extensive long-range interactions (Bantignies et al., 2011; Lanzuolo et al., 2007; Sexton et al., 2012; Tolhuis et al., 2011), suggesting an inverse correlation between long range interactions and PcG protein persistence through mitosis.

*H3K27me3 persists at PREs in the BX-C and in gene in G2/M cells.*

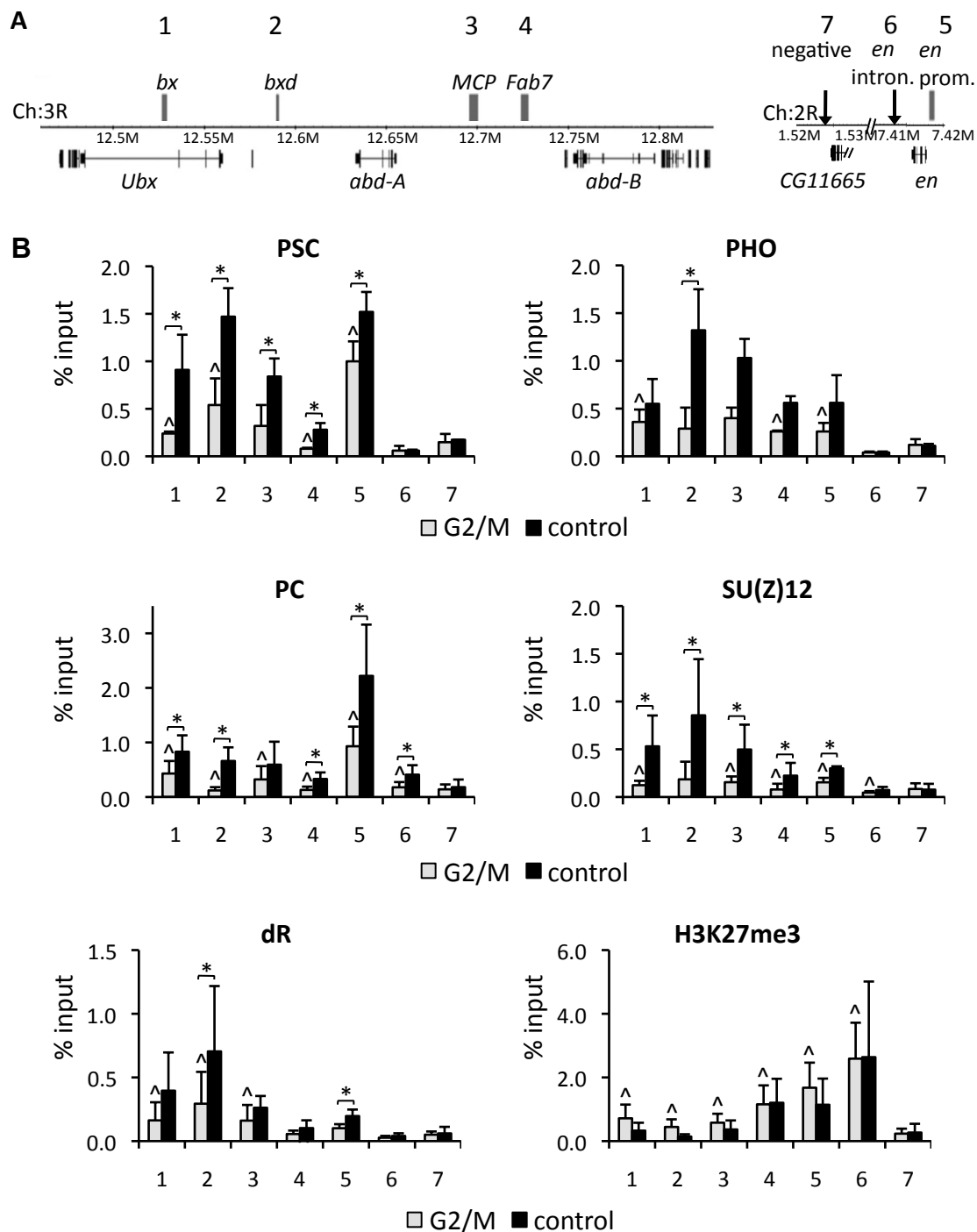
If PSC and PH binding is lost at target genes in mitosis, memory of repression may be carried by another PcG protein or the PcG-specific histone modification H3K27me3. To address this possibility ChIP assays were carried out on asynchronously growing (control) and colchicine-treated (G2/M) S2 cells. PSC, PC,



dR, SU(Z)12 binding and the PcG-specific histone modification H3K27me3 localization was analyzed for PREs in the BX-C and the *en* locus (Figure 3-7A). All PcG proteins and the H3K27me3 modification are bound at all target sites in control cells except for the *en* intron, at which only PC and H3K27me3 are bound, but not at a negative site (Figure 3-7B). In G2/M cells PSC binding is reduced at PREs, consistent with our analysis of FACS sorted pure mitotic cells (compare Figure 3-7B to Figure 3-1E) and indicating that binding detected at PREs in G2/M cells is due to the presence of G2 cells in the population. Association of PHO, PC, SU(Z)12, and dR with PREs is reduced in G2/M cells to a similar extent as PSC. Thus association of PHO, PC, SU(Z)12, and dR with these PREs is likely also lost in mitotic cells. In contrast, the H3K27me3 modification is present at comparable levels at PREs in control and G2/M cells, indicating that it most likely persists through mitosis.

## Discussion

How chromatin states can be propagated through mitosis is a major unanswered question in epigenetics. How PcG-dependent repression might be propagated through mitosis was investigated by systematically characterizing the behavior of PcG proteins during mitosis in *Drosophila* S2 cells. Using ChIP and ChIP-SEQ, it is shown here that PSC and PH are lost from the majority of their interphase binding sites during mitosis, but are retained at a small subset, including chromatin domain borders. These results suggest a model in which PSC and PH retained at



**Figure 3-7. PcG protein binding, but not H3K27me3, is reduced at PREs in the BX-C and *en* gene in G2/M cells.** (A) Schematic diagram of part of the BX-C and the *en* locus. (||) indicate PREs. Numbers indicate positions of primers used. (B) ChIP-qPCR for PcG proteins and H3K27me3 in control and G2/M cultures. \* $P < 0.05$  (two-tailed Student's *t*-test comparing control and G2/M). ^ $P < 0.05$  (two-tailed Student's *t*-test comparing G2/M to no antibody).

domain borders serve to nucleate re-binding of PcG proteins to target sites upon mitotic exit.

Our CHIP-SEQ data indicate that PSC/PH binding sites can be grouped into two classes: dynamic sites which lose binding of PSC and PH in mitosis, and persistent sites, at which these proteins are reduced but are clearly still present. The dynamic sites include well-characterized PREs controlling expression of important PcG targets like the *Hox* genes. The data thus provide clear evidence that propagation of PcG-dependent repression of most genes through mitosis does not involve persistent binding of PSC and PH to PREs near these genes. The CHIP data from G2/M cells also indicate that it is unlikely that any of the PcG proteins tested (PHO, PC, SU(Z)12) persist at *Hox* PREs. How then is PcG-dependent gene regulation propagated at these genes? The CHIP data suggest that H3K27me3 levels are unchanged in G2/M cells relative to controls, and thus that H3K27me3 is a candidate “epigenetic mark” at these sites in mitosis, which may work in conjunction with persistent sites (see below).

As discussed in Chapter 1, evidence exists both for and against the ability of H3K27me3 to recruit PcG proteins to target sites (Lee et al., 2007; Mujtaba et al., 2008; Platero et al., 1995; Schwartz et al., 2006). PREs are nucleosome-depleted and/or sites of high turnover, evidenced by nuclease hypersensitivity, enrichment in FAIRE, low H3 or H1 binding in CHIP or DamID experiments, and high levels of histone H3.3 (Braunschweig et al., 2009; Deal et al., 2010; Kahn et al., 2006; Kharchenko et al., 2010; Mishra et al., 2001; Mito et al., 2007; Mohd-Sarip et al.,

2006; Schuettengruber et al., 2009; Schwartz et al., 2006). If these structural alterations persist in mitosis, it is unlikely that H3K27me3 alone would be enough to re-target PcG proteins to PREs. The data presented here provide preliminary evidence that the chromatin structure at PREs remains disrupted in mitotic cells, since the ChIP signal for histone H3 is similar in both control and mitotic cells (Figure3-1E). In control cells, the H3 signal is low at these PREs (relative to nearby sites, not shown), consistent with other reports (Kahn et al., 2006; Mohd-Sarip et al., 2006; Papp and Muller, 2006). Thus, perhaps additional factors remain bound to these sites in mitosis.

A large fraction (~27%) of persistent mitotic PSC and PH binding sites overlap borders of large-scale chromatin domains. A possible model for the function of persistent binding sites is that they nucleate re-establishment of PcG binding at nearby sites. For example, persistent binding sites were not identified within the *Hox* gene clusters, but each cluster is flanked by persistent sites (Figure3-5F-H). PcG proteins bound at these flanking border sites during mitosis may nucleate recruitment of additional PcG proteins upon mitotic exit, creating loading sites from which the proteins might spread into the domain to PREs that are marked with H3K27me3 and possibly other persistent chromatin features. Interestingly, a careful study of PcG proteins through the cell cycle in mammalian cells indicated that PcG bodies, which are sites of long range interactions among PcG proteins in *Drosophila*, are reformed in G1 although some PcG proteins and H3K27me3 persist on chromosomes through mitosis (Aoto et al., 2008). A more recent study of PcG

proteins in *Drosophila* tissue culture cells also showed that PcG proteins increase at PREs in the Hox clusters towards the end of G1/early S phase (Lanzuolo et al., 2011), which could reflect post-mitotic re-establishment of PcG protein binding.

It is interesting that border regions are defined by their paucity of long range interactions, while many PcG sites (such as those in the *Hox* clusters) engage in extensive long range interactions (Bantignies et al., 2011; Lanzuolo et al., 2007; Sexton et al., 2012; Tolhuis et al., 2011). Perhaps long-range interactions are disrupted as cells enter mitosis, contributing to loss of PcG proteins. Regions of the chromosome that are not extensively networked with distal sites might undergo less disruption on entry into mitosis, which might allow PcG proteins to remain associated with them. Future investigation of long-range interactions in mitosis will clarify this point; preliminary data from G2/M cells suggests long-range interactions may be decreased in mitotic cells (Chiara Lanzuolo and Valerio Orlando, personal communication). An alternative model is that persistent binding is not related to re-establishment of binding at non-mitotic sites but instead reflects an independent function of PcG proteins, which might be related to organizing large-scale chromatin domains.

In summary, two key PcG proteins, PSC and PH persist on mitotic chromosomes. Persistent binding at specific sites during mitosis may nucleate re-establishment of repressive chromatin structures to propagate silencing through mitosis.

## Experimental Procedures

### *Cell culture and synchronization*

*Drosophila* S2 cells (Invitrogen, Carlsbad, CA) were cultured in ESF 921 media (Expression Systems, Woodland, CA) at a density between  $1$  and  $7 \times 10^6$  cells/mL in shaking flasks at 27°C. For synchronization, colchicine (Sigma) was added to cells to a final concentration of 350ng/ml (880nM). Cells were harvested 15 hours later by centrifugation at  $400 \times g$  for 5 min. at 4°C and then centrifuged twice ( $480 \times g$  for 5 min.) through a 20% sucrose cushion to remove cell debris. Aliquots of cells were collected, stained with propidium iodide and analyzed for DNA content on an LSRII cell sorter (BD Biosciences). Mitotic index was determined by staining an aliquot of cells with Hoescht and visually scoring cells with condensed chromosomes as mitotic. Similar results were obtained by staining cells with anti-H3S10p and carrying out FACS analysis.

### *Antibodies*

The affinity-purified anti-PSC antibody raised against PSC aa 521-869 was previously described (Francis et al., 2009). Antibodies against PC, PH, PHO, SFMBT and SU(Z)12 were kind gifts from J. Mueller. The antibody against dRING was a gift from R. Jones. The anti-CRM antibody was a gift from Walter Gehring, and the anti-dCBP antibody was a kind gift from Alexander Mazo. The anti-E(Z) antibody, (dL-19), and the anti- $\beta$ -tubulin antibody, (d-140) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The anti-H3 antibodies, ChIP-grade ab1791

(for ChIP) and ab39655 (for sorting), and the anti-H3K27me3 antibody, ChIP-grade ab6002, were purchased from Abcam (Cambridge, MA). The antibody to H3S10p was purchased from Millipore (Billerica, MA).

### *Cell sorting*

Colchicine treated or asynchronously growing S2 cells were fixed 1% formaldehyde for 10 min. at room temperature with rocking. glycine, pH 7.9 was added to 0.125mM to quench the formaldehyde. Cells were pelleted and centrifuged twice through a 20% sucrose cushion to remove cell debris. Cells were resuspended in 0.016% Triton-X 100, 1x PBS + protease inhibitors (which are used throughout) at a concentration of  $1 \times 10^7$  cells/mL and incubated for 15 min. on ice. Cells were pelleted and washed in 1% BSA, 0.1% Triton-X 100, 1x PBS and incubated with 2.7mg/mL FITC-conjugated anti-H3S10p antibody (colchicine-treated cells) or 3mg/mL anti-H3 antibody (control cells) at a concentration of  $1 \times 10^7$  cells/mL and incubated on ice in the dark for 30 min. Cells were pelleted and washed with 1% BSA, 0.1% Triton-X 100, 1x PBS. Control cells were incubated with FITC-conjugated secondary antibody for 30 min. and washed with 1% BSA, 0.1% Triton-X 100, 1x PBS. Cells were resuspended in 1x PBS, 10% horse serum and incubated overnight at 4°C in the dark. Cells were passed through a 40  $\mu$ M filter and sorted by the FAS Center for Systems Biology Flow Cytometry Core on a MoFlo Legacy Cell Sorter (Beckman Coulter) to collect FITC-positive cells. Cells were collected in 15mL glass tubes coated in horse serum. Pre- and post-sorted cell populations were analyzed on an

LSRII cell sorter (BD Biosciences). Sorted cells were counted, pelleted in  $4 \times 10^7$  cell aliquots, snap frozen in liquid  $N_2$  and stored at  $-80^\circ C$ .

### *Chromatin immunoprecipitation*

*Drosophila* S2 cells were fixed in 1% formaldehyde for 10 min. at room temperature with rocking. Glycine, pH 7.9 was added to 0.125mM to quench the formaldehyde. Cells were pelleted and washed with 1x PBS. Cells were counted and pelleted in  $1 \times 10^8$  cell aliquots, snap frozen in liquid  $N_2$  and stored at  $-80^\circ C$ .

Chromatin was prepared as follows, based on protocols from Upstate Biotechnology, Kind et al., 2008, Rastegar et al. 2004, and Schwartz et al. 2005. Pellets were thawed and resuspended in wash buffer I (10mM HEPES, pH 7.6, 10mM EDTA, 0.5mM EGTA, 0.25% Triton X-100, plus protease inhibitors as described above), incubated 10 min. at  $4^\circ C$  with rotation, centrifuged ( $250 \times g$ , 4 min.,  $4^\circ C$ ) and resuspended in wash buffer II (10mM HEPES, pH 7.6, 200mM NaCl, 1mM EDTA, 0.5mM EGTA, 0.01% Triton X-100, plus protease inhibitors as described above), incubated 10 min. at  $4^\circ C$  with rotation and centrifuged ( $250 \times g$ , 4 min,  $4^\circ C$ ). Pellets were resuspended in sonication buffer (50mM HEPES, pH 7.5, 500mM NaCl, 1mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS) to a cell concentration of  $2 \times 10^7$  cells/mL in 2 mL and sonicated with 10\*30 second pulses with 30 seconds between pulses using a Sonics Vibracell sonicator at 40% power. Sonications were checked by agarose gel electrophoresis, and fragment sizes were centered on 450 bp. Following sonication, samples were centrifuged for 5 min. at full speed in a refrigerated microcentrifuge.



The supernatant was used for ChIP.

100  $\mu$ L of chromatin (corresponding to  $\sim 2 \times 10^6$  cells) were used for each reaction and were adjusted to 1x ChIP binding buffer (15mM Tris, pH 8, 150mM NaCl, 1mM EDTA, 1% Triton X-100, 0.01% SDS). For Protein A-agarose-ChIP, samples were pre-cleared with Protein A agarose beads (Sigma) which were blocked with BSA and salmon sperm DNA (Invitrogen) (for 5 ml of packed beads: 5 mL of 10mM Tris, pH 8.0, 1mM EDTA, 0.05% sodium azide; 2-4mg salmon sperm DNA, 5mg BSA). For biotin-ChIP samples were pre-cleared with magnetic Dynabeads M-280 streptavidin (Invitrogen) blocked with 0.2mg/mL salmon sperm DNA. For biotin-ChIP both antibodies were biotinylated using a kit according to the manufacturer's instructions (Anaspec or Thermo-Pierce). 2 $\mu$ g of unbiotinylated or biotinylated antibody was used, which in titration experiments was shown to be saturating, and 0.5 $\mu$ g of anti-H3 was used. The next day, Protein A-agarose or magnetic beads were added to capture immune complexes for 1-2 hour at 4°C. Agarose beads were washed once for 5 min. at 4°C with rotation in each of the following buffers: low salt (20mM Tris, pH 8.0, 0.1% SDS, 1% Triton X-100, 2mM EDTA, 150mM NaCl), high salt (same as low salt but 500mM NaCl), lithium chloride (0.25M lithium chloride, 10mM Tris, pH 8.0, 1% NP40, 1% deoxycholate, 1mM EDTA) followed by 2 washes in TE, pH 8.0. Magnetic beads were washed 3x in ChIP binding buffer for 5 min. at RT with rotation. Agarose beads were isolated by centrifugation at  $106 \times g$  for 1 minute, while magnetic beads were isolated on a magnetic rack for 2 min. To elute DNA from agarose beads, beads were incubated with agarose elution buffer (1% SDS, 0.1M

sodium bicarbonate) twice for 15 min. at room temperature. Elutions were pooled and adjusted to 200mM NaCl. DNA was eluted from magnetic beads by addition of biotin elution buffer (1% SDS, 0.5M NaCl). Elutions were incubated at least 6 hours at 65°C to reverse crosslinks. Samples were treated for 30 min. with RNase A at 37°C and 1 hour with Proteinase K at 55°C, and DNA was purified with a Nucleospin Extract II kit (Macherey-Nagel).

qPCR was carried out on a Bio-Rad IQ5 machine using SYBR green (Bio-Rad). 1:5 and 1:10 dilutions of ChIP DNA and 1:100 and 1:200 dilutions of input DNA were used to ensure reactions were in the linear range. Standards ranged from 100ng to 0.1ng of genomic DNA from S2 cells and 40 cycles of PCR were run. Detection was carried out at 76°C.  $R^2$  values for the standard curves were all above 0.9.

#### *Genome-wide sequencing and data analysis*

6-50ng of input or immunoprecipitated DNA were submitted to the BioMicro Center (MIT, Cambridge, MA) for library generation and sequencing on an Illumina HiSeq 2000. Two sets of samples were first fragmented using a Bioruptor 300 (Diagenode). 25ng of DNA was biorupted for 80 pulses on the low setting to generate fragments ~180bp. 36-bp reads were aligned to the *D. melanogaster* genome (dm3) using Bowtie 1.1.2 (Langmead et al., 2009) through Galaxy (Blankenberg et al., 2010; Giardine et al., 2005; Goecks et al., 2010) retaining uniquely mapping reads with up to 2 mismatches in the first 28 bp. Binding sites were identified using MACS through Galaxy using fragment size 36, genome size

120,000,000, bandwidth 200, cutoff P-value  $<10^{-5}$ . The results were filtered to retain peaks with a false discovery rate of 5% or below for all samples except for mitotic PH. Peaks on chromosome U and Uextra were removed. Pearson's correlation coefficient was used as a measure of correlation between datasets and was computed for number of reads within each peak for all called peaks in either of the sets. Heatmaps were generated using seqMINER 1.2.1 (Ye et al., 2011). Average profile plots and distribution analysis were done with CEAS 1.0.2 (Shin et al., 2009).

#### *Comparison with other datasets*

ChIP-chip tiling array data and ChIP-SEQ data were downloaded from the modENCODE consortium (modMine.org), GEO ([www.ncbi.nlm.nih.gov/geo/](http://www.ncbi.nlm.nih.gov/geo/)), or supplemental material as available (Schwartz et al., 2006). Called peaks from the original studies were used to retain consistency with published work. Overlaps and enrichments were calculated using BEDtools 2.12.0. Enrichment analysis was performed as in (Moshkovich et al., 2011). The matrix of pairwise enrichment scores was constructed using GiTools (Perez-Llamas and Lopez-Bigas, 2011).

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## Chapter 4

### Methods for isolation of *Drosophila* tissue culture cells from different stages of the cell cycle

A description of the methods presented in this chapter have been accepted for publication.

Follmer, N.E. and Francis, N.J. (2012). Preparation of *Drosophila* tissue culture cells from different stages of the cell cycle for chromatin immunoprecipitation using centrifugal counterflow elutriation and fluorescence-activate cell sorting. Methods in Enzymology. (Accepted for publication.)

## Abstract

Many nuclear proteins alter their localization during the cell cycle. This includes proteins that regulate and execute cell cycle events and proteins involved in transcription and DNA repair. The core components of chromatin, the histone proteins, also change their modification state through the cell cycle. Chromatin immunoprecipitation (ChIP) makes it possible to localize chromatin-associated proteins to specific sequences in the genome and has revolutionized studies of transcription. Fewer studies have used ChIP to analyze protein localization or modification at specific stages in the cell cycle. This is in part because these studies require isolation of pure populations of cells at each stage of the cell cycle, which is challenging for many cell types. However the ability to carry out ChIP from cells at specific stages in the cell cycle in some systems has revealed cell cycle regulation of chromatin localization and cell cycle stage-specific functions and modification of chromatin proteins, providing incentive to pursue these experiments. We have adapted existing methods to isolate *Drosophila* S2 cells suitable for ChIP analysis from all phases of the cell cycle using centrifugal counterflow elutriation and Fluorescence Activated Cell Sorting (FACS).

## Introduction

Chromatin immunoprecipitation is a powerful tool for localizing chromatin-bound proteins. Localization of proteins to particular sites on chromatin can be queried by qPCR, or microarray or high-throughput sequencing can be used to reveal

global localization of a protein within the genome. Localization of many proteins to chromatin varies through the cell cycle. This includes proteins involved in DNA replication and mitotic chromosome condensation, but likely also includes many proteins that regulate transcription. ChIP on pure populations of cells from specific stages of the cell cycle is a powerful way to study cell cycle-dependent chromatin protein dynamics (Blobel et al., 2009; and studies reviewed in Young and Kirchmaier, 2011). In some cells, such as yeast, cell cycle-staged populations can be achieved with simple, well-characterized treatments (Fox, 2004; Manukyan et al., 2011). In other cell types, such as *Drosophila* tissue culture cells, cell cycle-synchronizing drugs work inefficiently. Thus other methods must be found to obtain pure populations of cells from the different stages of the cell cycle.

Centrifugal counterflow elutriation is a method for separating heterogeneous cell populations by sedimentation rate, a property that depends in part on the diameter and density of the cell. As cell size correlates well with DNA content and thus with the cell cycle, this method can separate an asynchronously growing population of cells according to cell cycle stage. Cells are placed in a specialized rotor in which centrifugal force is balanced by counterflow of buffer or media. Stepwise increase in flow rate or decrease in centrifugal force allows for separation of the heterogeneous cell population into successive fractions of uniform size, starting with the smallest, most slowly sedimenting cells and ending with the largest, most rapidly sedimenting cells. This method allows for the separation of cells into G1, S and G2 populations without the use of pharmacological or other perturbations

of the cells. While centrifugal counterflow elutriation can be performed on living cells, the method presented here is for the separation of formaldehyde fixed cells, which are suitable for chromatin immunoprecipitation.

Mitotic cells do not fractionate as a single population via centrifugal elutriation (Hengstschlager et al., 1997; Kauffman et al., 1990), thus other methods must be employed to obtain pure populations of mitotic cells in large quantities. Only a small percentage of cells in an asynchronously growing culture are in mitosis (4-5% for *Drosophila* S2 cells), so that large-scale separation by any means is challenging. Mitotic shake-off, a method used for many adherent cell lines to obtain purely mitotic populations (Terasima and Tolmach, 1963), is not feasible in *Drosophila* S2 cells, or other cell types that grow in suspension or semi-adherent. Mitotic-arrest inducing drugs can be used, but the cells respond with incomplete mitotic arrest. A solution arises with a combination of strategies: arrested mitotic cells can be separated from the non-mitotic cells in a drug-treated culture with the use of a mitotic marker in conjunction with flow cytometry.

This chapter describes methods for isolating *Drosophila* S2 cells from each stage of the cell cycle that are suitable for ChIP. G1, S, G2 cells are isolated by centrifugal elutriation, while mitotic cells are isolated by FACS.

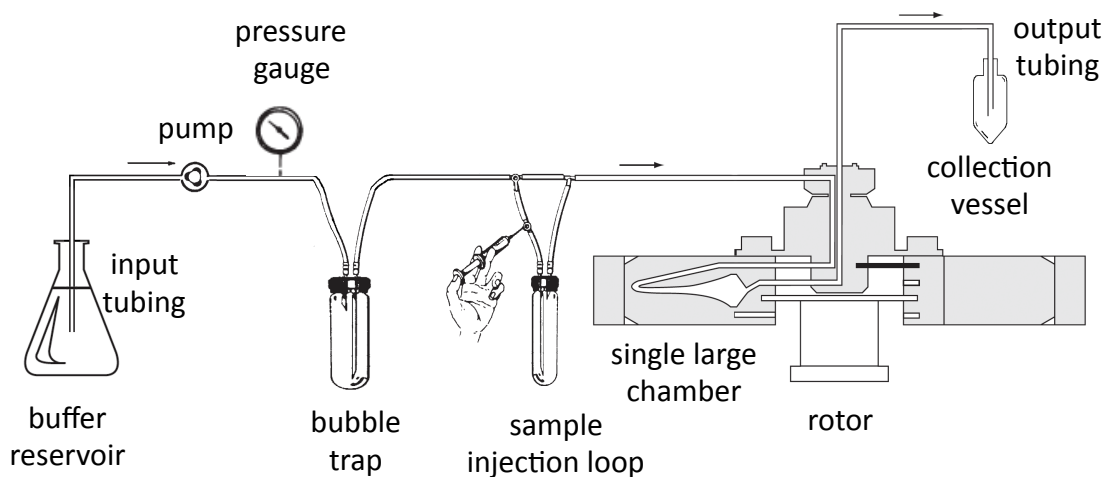
## Results

*Centrifugal Elutriation of Drosophila S2 cells generates relatively pure populations of G1, S and G2 cells.*

A method for isolating G1, S and G2 populations of fixed *Drosophila* S2 cells using centrifugal elutriation was developed, loosely based on (Banfalvi et al., 2007). The method was optimized using one large chamber (40mL) with a counterbalance in a JE-5.0 rotor in an Avanti J-26XP centrifuge (Beckman Coulter). The system is set up as in Figure 4-1. Critical parameters were found to include: flow rate, centrifuge speed, amount of time cells spend equilibrating in the chamber prior to elution, total cell number and fraction size. An optimized elutriation scheme is presented in Table 4-1. Using this method asynchronously growing populations of S2 cells with starting cell cycle composition of approximately 15% G1, 50% S, 30% G2 and 5% M were separated into relatively pure G1, S and G2 populations. Purity of such fractions range from 75-85% G1, 100% S and 85-92 %G2. S-phase can be further subdivided into early, mid, and late S-phase. Representative results are shown in Figure 4-2. Roughly 20-30% of the total number of cells in each phase are recovered in these highly-enriched fractions.

*PSC levels at Polycomb Response Elements (PREs) are reduced after S phase.*

The enriched cell populations generated by elutriation are suitable for ChIP. These cell populations were used for ChIP to localize PSC to PREs in the Bithorax Complex (BX-C) and at the *engrailed* locus, as shown in Figure 4-3. These experiments show that the percentage of PSC pulled down at each PRE decreases as the cell cycle progresses from G1 through S and into G2 (Figure 4-3C). Comparison of PSC levels at each of these sites in G1 vs. G2 by paired Student's *t*-



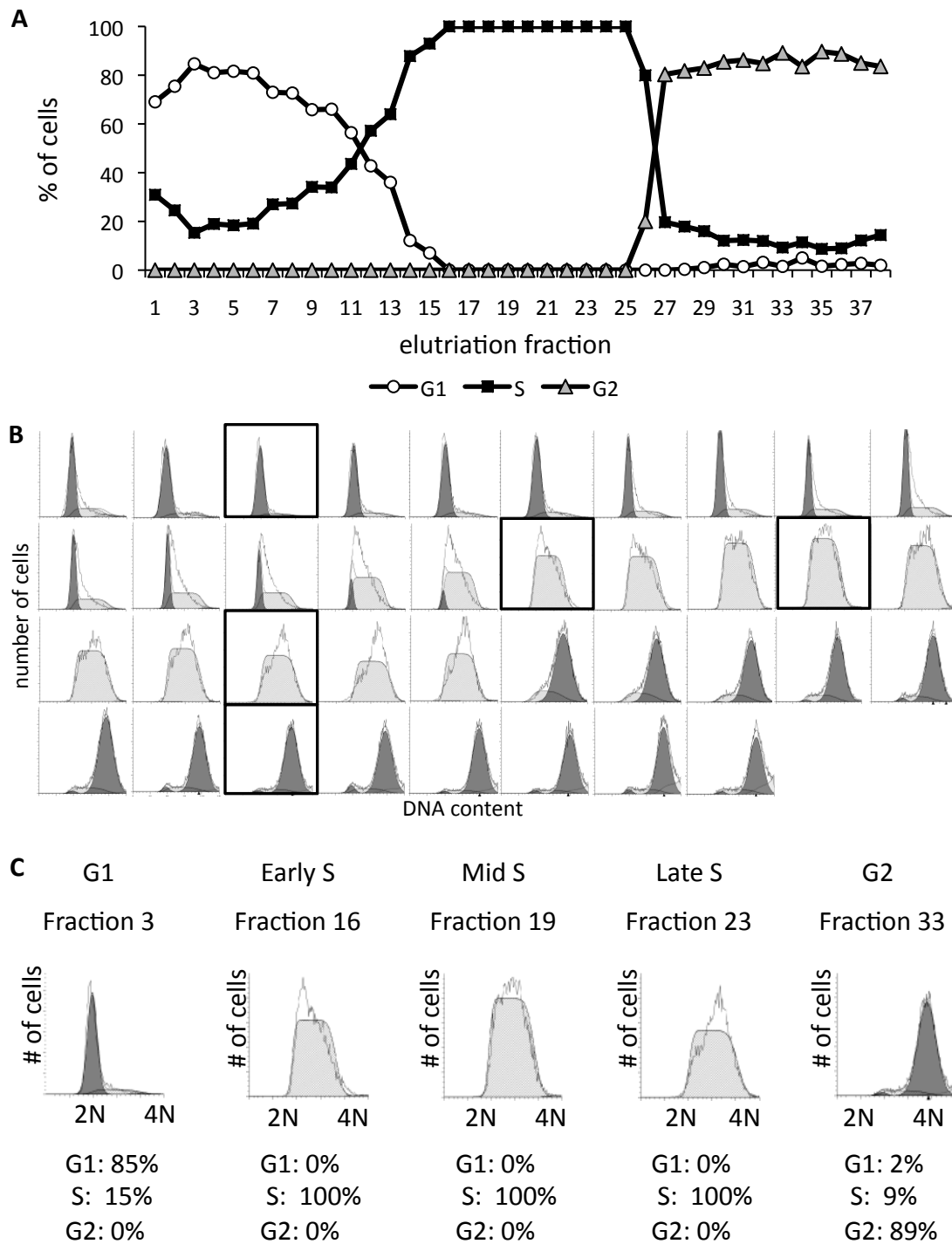
**Figure 4-1. The elutriation system.** A 4L Erlenmeyer flask serves as a buffer reservoir. A variable-speed pump is placed between the buffer reservoir and a bubble trap, and a pressure gauge is placed between the pump and the bubble trap. A sample injection loop follows the bubble trap and consists of: a 3-way valve to divert flow through the injection loop or to allow flow to bypass the loop, a 3-way valve for syringe attachment for sample injection, and a sample reservoir. The tubing then passes into the rotor, through a single large chamber and out to a collection vessel. Image adapted from Beckman Coulter (2007-2009). Figure 2-6. Images courtesy of Beckman Coulter, Inc.

The JE-5.0 Elutriation System is for Laboratory Use Only. Not for use in diagnostic procedures.

**Table 4-1. Optimized elutriation scheme.**

starting and equilibration centrifuge speed	3,500 rpm (2,300 x <i>g</i> )
number of cells loaded	1-1.5x10 <sup>9</sup> cells
loading flow rate	80mL/min.
equilibration flow rate	96mL/min.
equilibration time	2 hours
fraction volume	200mL
fractions per centrifuge speed increment	2
centrifuge speed decrease per 2 fractions	50 rpm (0.5 x <i>g</i> )
number of fractions collected	44

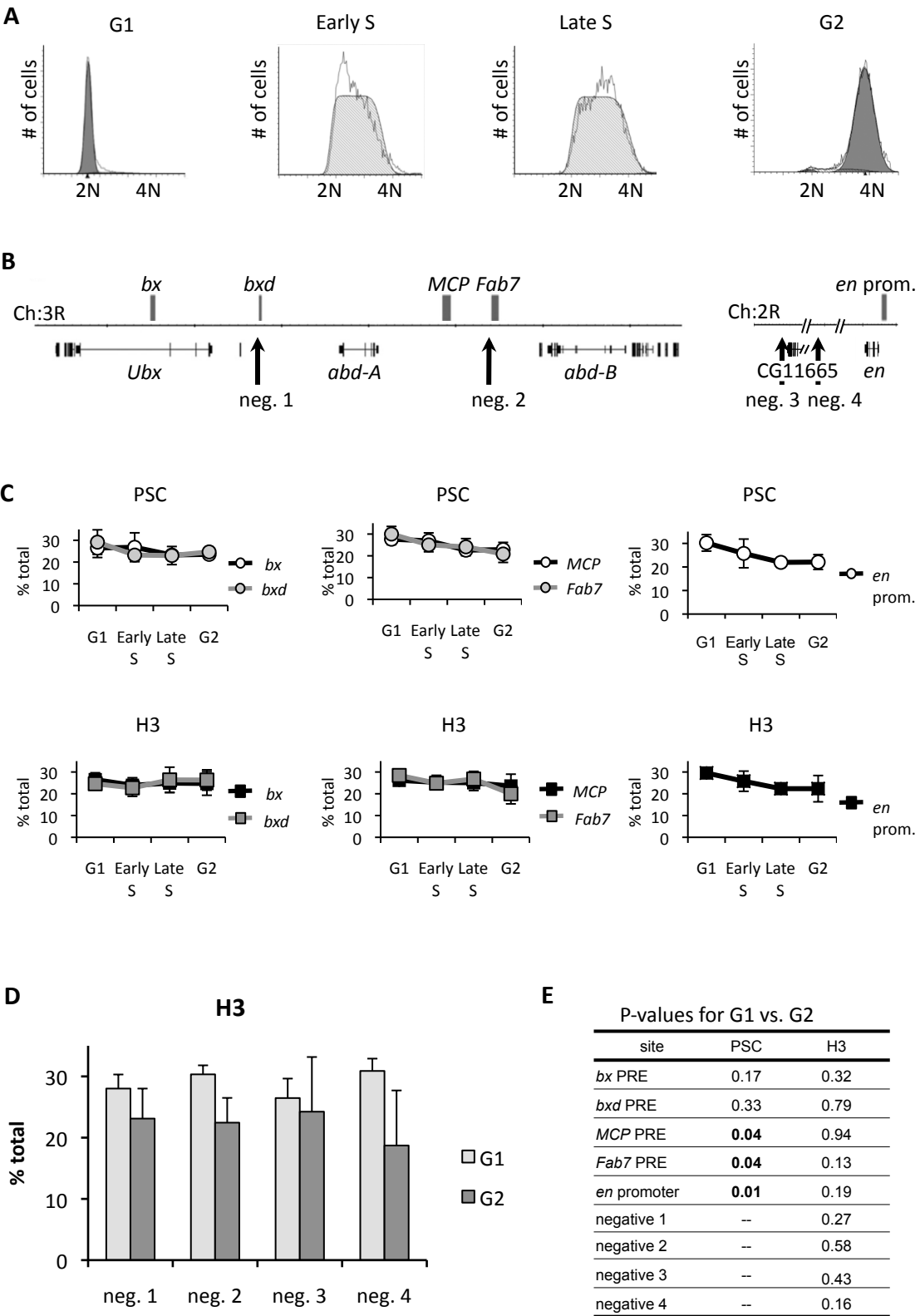




**Figure 4-2. Representative elutriation profile.** (A) Summary graph of percentage of cells in G1, S or G2 for each fraction of a representative elutriation. (B) FACS profiles of all fractions of the elutriation. Black boxes indicate fractions containing relatively pure populations of G1, S or G2 cells, displayed in C. (C) Cell cycle analysis of fractions containing G1, early S, mid S, late S or G2 cells.

**Figure 4-3. PSC association with some Polycomb Response Elements (PREs) is reduced in G2 relative to G1.** (A) Representative FACS profiles of fractions of elutriated cells used for analysis of PSC binding to chromatin in G1, early S, late S, and G2. (B) Schematic of PREs used for analysis (Francis et al., 2009). (C) Average profile of PSC and H3 across the cell cycle for 5 PREs. ChIP experiments were expressed as % input; the graphs show the fraction of the signal from all 4 samples contributed by each sample (e.g. G1/total); if the levels were constant each number should be 25%. To assess possible differences in the quality of chromatin isolated from cells at different stages of the cell cycle, ChIP was also carried out for histone H3. (D) Summary of histone H3 ChIP in G1 vs. G2 at 4 non-PRE sites. (E) Table of P-values for paired Student's *t*-test analysis of PSC or H3 associated with PRE and non-PRE sites in G1 versus G2; significant values ( $P < 0.05$ ) are in bold. In some cases, histone H3 signals were reduced between G1 and G2 as well, but none of the differences between G1 and G2 were significant for H3 at PREs, or at any of 4 other non-PcG binding sites.

**Figure 4-3 (Continued)**

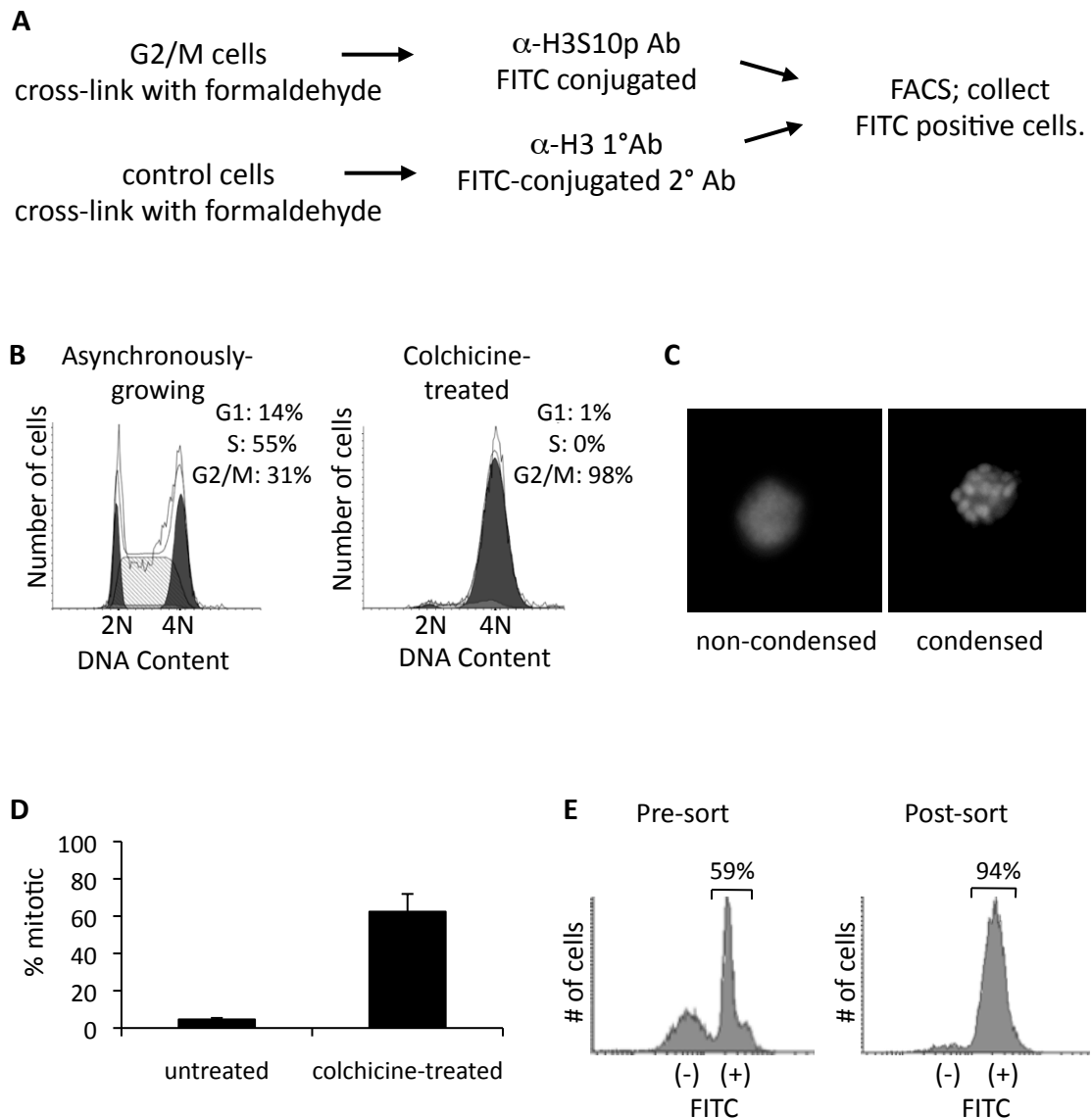


test showed that for 3 of the 5 sites tested, PSC association is statistically significantly decreased in G2 vs. G1 (Fig. 4-3E), while H3 occupancy at these sites and at a number of negative sites (Figure 4-3D) was not. Thus PSC levels at some target genes are reduced in G2 vs. G1.

#### *FACS sorting of Drosophila cells to obtain mitotic cell populations*

A method for isolating mitotic populations of fixed *Drosophila* S2 cells was devised using Fluorescence Assisted Cell Sorting (Figure 4-4A). Cells are treated with colchicine which results in a G2/M population with ~60-66% cells arrested in mitosis (Figure 4-4D, E). The cells are then immunostained for an intracellular mitotic marker, phosphorylated histone H3 (H3S10p), using an antibody that is conjugated to FITC. The stained cells are sorted, and FITC-positive cells are collected. In parallel asynchronous cultures are stained with an antibody for histone H3, and a FITC conjugated secondary antibody is used. The stained cells are sorted, and FITC-positive cells are collected.

This method allows for the isolation of > 95% pure mitotic cells (Figure 4-4E) in quantities sufficient for ChIP studies. Representative recovery of sorted cells (number of cells recovered/total cells sorted) range from 11-45%, with an average recovery of 23%. Cells recovered as a percentage of flow cytometer counts is > 85%, averaging 98%. The average sorting rate is  $29 \times 10^6$  cells/hr, while the average recovery rate is  $7 \times 10^6$  cells/hr.



**Figure 4-4. Colchicine treatment and FACS profiles.** (A) Schematic diagram of the FACS sorting protocol to isolate mitotic cells based on H3S10p immunoreactivity. Control cultures were sorted with antibodies to histone H3. (B) Representative FACS profiles of asynchronously growing and colchicine treated cells. (C) Images of Hoechst-stained colchicine treated cells showing condensed, mitotic (right) and non-condensed non-mitotic cells (left). (D) Quantification of Hoechst-stained cells with condensed chromosomes from untreated or colchicine treated cultures. (E) FACS profiles of pre- and post-sorted colchicine-treated cells.

*FACS sorted S2 cells are suitable for ChIP*

ChIP performed with sorted cells is performed with biotinylated antibodies and streptavidin coated beads rather than Protein A or G beads to avoid pulling down the antibodies used for sorting. Experiments using this method are presented in Chapter 3.

## **Discussion**

Here methods are described for isolation of *Drosophila* S2 cells from every stage of the cell cycle suitable for chromatin immunoprecipitation. G1, S phase and G2 cells are separated by centrifugal counterflow elutriation, while mitotic cells are isolated by FACS sorting. These methods were used to study PcG proteins throughout the cell cycle. Results describing the behavior of PSC in cells in G1 through G2 are presented in this chapter, while the study of PSC and PH in mitotic cells isolated using the methods in this chapter was presented in Chapter 3.

The methods presented in this chapter should have broad applicability for the study of chromatin-binding proteins during the cell cycle. Certainly these methods can be used to study other *Drosophila* proteins in S2 cells by chromatin immunoprecipitation, but the methods may also be used in other contexts. For example, fixed cells from each stage of the cell cycle could be used for purposes other than ChIP. The use of formaldehyde, a reversible crosslinker, could allow for the extraction of RNA, DNA or protein from the cell cycle separated cells generated by the methods described here, following crosslink reversal. The parameters

described for the separation of S2 cells by elutriation are likely not directly applicable to other cell lines, given the dependence of the method on the physical characteristics of the cells, but they could be used to separate out live S2 cells as a drug-free method for synchronizing cultures. The applicability for the isolation of mitotic cells is potentially much more broad. It could prove useful for any cell line that does not respond well to cell cycle arresting drugs and cannot be easily separated by physical means. The sorting procedure could also be performed as a drug-free alternative for obtaining purely mitotic cultures by performing it without pretreatment with mitotic arrest inducing drugs, although the relatively low yield of the sorting procedure may prove this route prohibitive in many cases.

The results obtained for the localization of PSC to PREs in the BX-C in G1, S and G2 cells indicate that PSC binding at these sites decreases from G1 through S to G2. It has been shown that PSC is transferred from parental DNA strands to newly replicated strands of DNA *in vitro* and is bound to newly replicated chromatin in S2 cells (Francis et al., 2009--Appendix), which establishes the basis of a model by which PcG-dependent repression can be maintained through DNA replication. The decrease in binding seen here is consistent with the dilution of PSC at some PREs as the genome is duplicated through DNA replication. The difference in G1 vs. G2 binding also suggests that additional PSC is recruited in G1, relative to G2. These results contrast with a recent report in which PcG proteins (other than PSC) are enriched at PREs in early S phase relative to either cells blocked at the G1/S border or cells in late S (Lanzuolo et al., 2011). In those data the difference in PcG protein

occupancy of PREs between early S and G1/S cells or cells in late S is much larger than the difference in PSC occupancy at PREs observed here. The decrease Lanzaolo *et al.* see in PcG protein occupancy at PREs between early and late S may also support dilution of the proteins as the loci are replicated, although the difference is much greater than two-fold. The increase in binding in early S relative to cells at the G1/S border observed by Lanzaolo *et al.* supports a model in which additional PcG proteins are loaded early in S-phase, rather than in G1, as described here. These differences could be explained by a difference in behavior in the proteins tested in that study vs. PSC examined here. Alternatively it is possible that the difference is in cell treatment (Lanzaolo *et al.* use hydroxyurea to block the cell cycle vs. the elutriation used here) is the cause of the differences seen.

## **Experimental procedures**

### *Cell culture conditions*

*Drosophila* S2 cells are grown in shaking flasks at 27°C at a density between 2 and  $10 \times 10^6$  cells/mL in protein-free insect cell media containing 34 mM L-glutamine.

### **Centrifugal Elutriation**

#### *Preparing the elutriator for a run*

Tubing running through the pump is checked to make sure it is not worn. If it is worn, an unworn length of tubing is placed through the pump. The elutriation is carried out in a centrifuge set to 4°C. Input tubing is placed in the 4L buffer reservoir



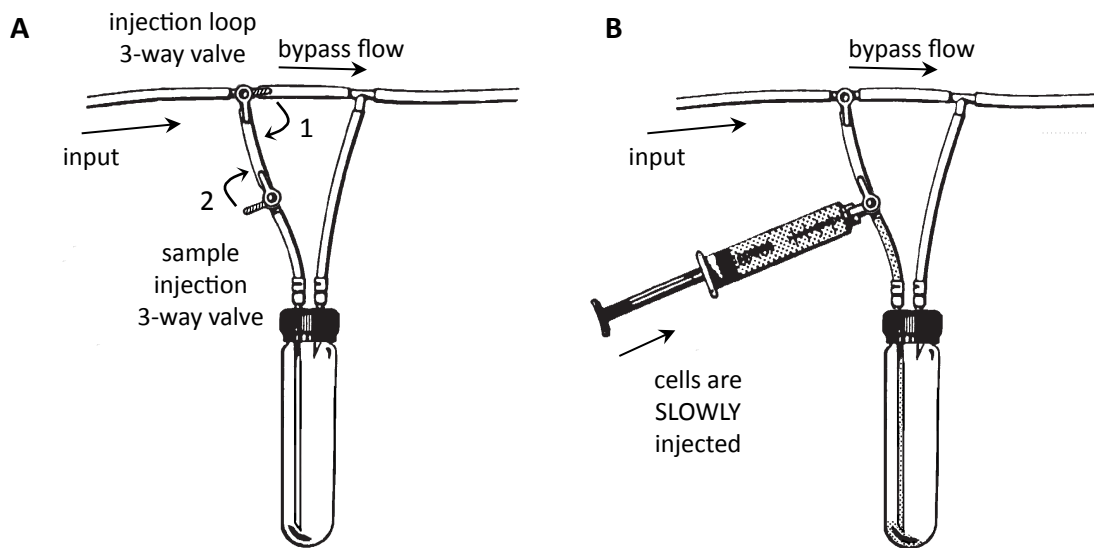
containing cold elutriation buffer (1x PBS, 5% horse serum). Air is purged from the system with the pump set to 200 mL/min. The bubble trap is inverted to allow it to fill with buffer and the valve to injection loop is opened to allow it to fill with buffer. With the pump running the centrifuge is set to 1,000 rpm (880 x *g*) to allow air to be purged from the elutriation chamber. Centrifuge speed is increased to 3,500 rpm (2,300 x *g*) to test for an imbalance. If an imbalance error occurs, the centrifuge is stopped and the quick-release assembly containing the elutriation chamber and balance is re-seated. Before each run, pump calibration is checked and reset if necessary. Centrifuge speed is set to the starting speed of 3,500 rpm (2,300 x *g*) and pump speed is set to the starting flow rate of 80mL/min. Notes: While counterbalance between flow rate and centrifugal force determines whether or not particles will be retained in the rotor, higher flow rates and faster centrifuge speeds afford greater resolution of separation.

#### *Loading the cells and equilibration*

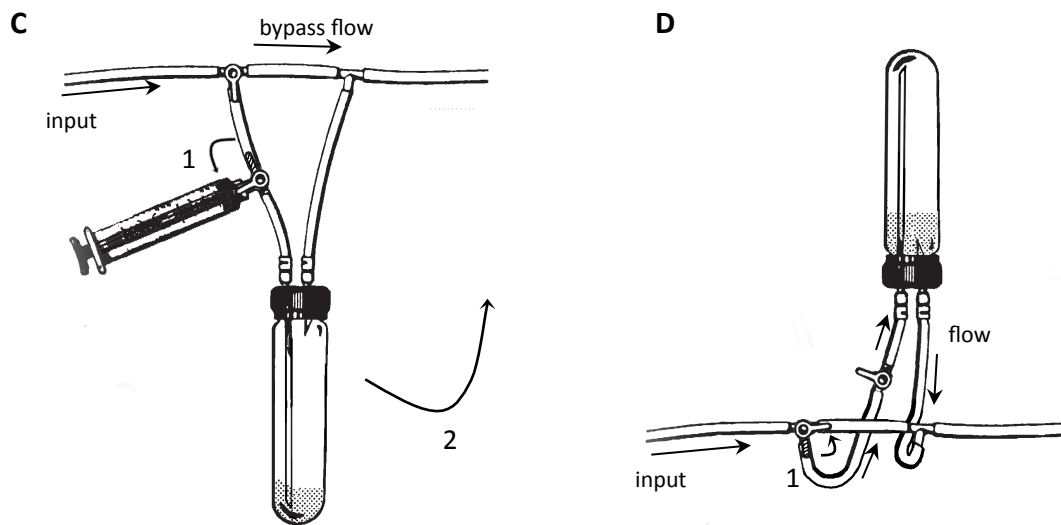
1-1.5x10<sup>9</sup> cells are fixed by adding formaldehyde directly to the tissue culture media to a final concentration of 1% and are rocked at room temperature for 10 minutes. Formaldehyde is quenched by adding 2.5M glycine, pH 7.9 to a final concentration of 130 mM. Cells are centrifuged for 5 min. at 4°C at 1,920 x *g* and resuspend in 1x PBS to wash. 1.5x10<sup>6</sup> cells are removed for FACS analysis. The rest of the cells are centrifuged for 5 min. at 4°C at 1,920 x *g*. Cells are then resuspended in elutriation buffer to a final volume of 10mL. Cells are passed slowly through an 18

gauge needle attached to a 10mL syringe ten times to disperse cell clumps, with care taken not to introduce air bubbles into the cell solution. The syringe is filled with the cell solution and the needle is removed.

To introduce the cells into the elutriation system, the injection loop three-way valve is closed so that the buffer bypasses the injection loop. The sample injection three-way valve is closed to the input (Figure 4-5A). Back pressure should push some buffer out of the side Luer fitting, purging air from the valve. The syringe containing the cells is attached to the side Luer fitting of the sample injection three-way valve and the cells are slowly injected into the sample reservoir (Figure 4-5B). Cells collect at the bottom of the sample reservoir. The valve is closed to the syringe. The sample reservoir is slowly inverted to allow the cells to move as a bolus to the top of the sample reservoir where the output is (Figure 4-5C). The output tubing is placed into the first collection tube (225mL conical tubes placed on ice) and the injection loop three-way valve is opened to the injection loop to allow the cells to move out of the sample reservoir into the rotor (Figure 4-5D). After all cells have passed out of the injection loop, the injection loop three-way valve is closed so that buffer bypasses the injection loop. The cells in the elutriation chamber are examined--a clear front of cells is visible near the top of the chamber. Note: The appearance of swirling of the cells, known as the Coriolis jetting effect, is undesirable. This causes mixing of cells in the chamber, which decreases the resolution of separation. Increasing the number of cells loaded into the chamber greatly decreases the Coriolis jetting effect.



1. Injection loop is closed to allow bypass.
2. Valve is closed to input to open to side Luer fitting and sample reservoir.



1. Sample injection valve is closed to syringe.
2. Sample reservoir is slowly inverted.
1. Valve is opened to injection loop.

**Figure 4-5. Loading cells into the elutriation system.** The steps of loading the cells into the elutriation chamber are illustrated here. **(A)** The injection loop is closed to buffer flow. **(B)** Cells are slowly injected into the sample reservoir. **(C)** The sample injection valve is closed to the syringe and the sample reservoir is slowly inverted. **(D)** The valve is opened to the injection loop to allow cells to enter the chamber. Images adapted from Beckman Coulter (2007-2009).

Over the first two 200mL fractions pump speed is incrementally increased to 96mL/min, i.e. pump speed is increased to 84mL/min. after 100mL has been collected, to 88mL/min. after 200mL has been collected, to 92mL/min. after 300mL has been collected and up to 96mL/min after 400mL has been collected. The first two fractions only contain cell debris and a few ( $< 1 \times 10^6$ ) very small cells. Note: If cells are escaping the chamber during the equilibration, either 1) the chamber is overloaded or 2) the centrifugal and counterflow forces are not balanced. If the latter is the cause either the flow rate is too high, or the centrifuge speed is too low. The flow rate is set as high as possible to avoid pelleting during equilibration while still retaining the smallest cells in the chamber. If the cells pellet during equilibration the pump speed is too low or the centrifuge speed too high.

Cells are left to equilibrate at 96 mL/min. 3,500 rpm (2,300 x g) for 2 hrs. To insure that cells are not escaping the chamber during the equilibration, the first several 200ml fractions are collected and monitored during the equilibration. Fractions are centrifuged at 4°C at 1,920 x g for 5 min., and the supernatant is gently poured off. The pellet is resuspended in 1 mL of cold 1x PBS. A sample of each resuspended pellet is examined on a hemocytometer under a light microscope to verify the absence of cells. If no cells are present, the rest of the fractions collected during the 2 hr. equilibration are passed through a 0.2  $\mu$ m filter and reused.

*Collecting and processing fractions*

All fractions are collected and processed on ice. All centrifugation is carried out at 4°C. To begin collecting the cells after the equilibration, the rotor speed is decreased by 50 rpm (0.5 x *g*) and two 200 mL fractions are collected. Rotor speed is continued to be decreased by 50 rpm (0.5 x *g*) increments, collecting two 200mL fractions at each step, until the rotor speed is 2,200rpm (910 x *g*). This will be 44 post-equilibration fractions. Cells are collected from each fraction by centrifugation for 5 min. at 1,920 x *g* in a swinging bucket rotor. The supernatant is gently decanted, taking care not to disrupt the cell pellet. Cells are resuspended in 2-15mL of ice cold 1x PBS. Note: Fractions are processed as the elutriation run is in progress. The elutriation centrifuge is situated near the low speed centrifuge used to pellet the fractions so that one person can both process the fractions and monitor the elutriation. Supernatants from fractions after centrifugation are filtered through a 0.2 µm filter and reused.

The volume for each fraction is recorded and cells are counted using a hemocytometer to determine the total number of cells in each fraction.  $1.5 \times 10^6$  cells per fraction are removed for FACS analysis. Cells in each fraction are collected by centrifugation for 5 min. at 1,920 x *g* in a swinging bucket rotor. The supernatant is removed and the pellets are flash frozen in liquid nitrogen and stored at -80°C.

#### *FACS analysis of fractions*

All samples saved for FACS analysis (including the input) are pelleted by centrifugation for 5 min. at 1,920 x *g* in a swinging bucket rotor at 4°C. Pellets are

resuspended in 100 $\mu$ L 1x PBS, and 900 $\mu$ L ice cold 95% ethanol is added. Samples are incubated at -20°C for  $\geq$  30 min to fix the cells. Fixed cells are pelleted by centrifugation in a swinging bucket rotor for 5 min. at 560 x *g*. Note: It is important to spin the ethanol fixed cells in a swinging bucket rotor to avoid damaging them against the side of the microfuge tube.

Pelleted cells are resuspended in 1x PBS with 5  $\mu$ g/mL propidium iodide and 50  $\mu$ g RNase A. Samples are incubated for 2 hrs. at room temp. Note: It is important to allow the RNase treatment to go to completion. Incomplete RNA degradation results in a contribution of RNA to the FACS profile of propidium iodide stained cells, which may render the cell cycle analysis uninterpretable. In this case, samples can be saved and RNase treatment repeated.

Samples are analyzed immediately or stored at 4°C up to 3-4 days. Samples are analyzed on a flow cytometer (i.e. LSRII cell sorter, BD Biosciences) and cell cycle analysis on the FACS profiles is performed using appropriate software (i.e. Modfit).

### **Colchicine treatment and FACS sorting**

#### *G2/M arrest with colchicine-treatment*

Colchicine is added to cells to a final concentration of 350ng/mL (880nM). The cells are at a concentration of  $\geq 5 \times 10^6$  cells/mL. 15 hours later, two  $1.5 \times 10^6$  cell aliquots are taken out for FACS analysis of DNA content and to count condensed chromosomes. Note: Drug concentration, time of treatment and cell density may

have to be titrated to obtain optimal arrest. These conditions reproducibly yield 60-66% mitotic cells (Figure 4-4B, D).

Cells are fixed with 1% formaldehyde in media for 10 min., rocking or shaking at room temperature. Formaldehyde is quenched by adding 2.5M glycine pH 7.9 to a final concentration of 130mM. Cells are centrifuged at  $730 \times g$  at  $4^{\circ}\text{C}$  for 5 min. and resuspended in ice cold 1x PBS to a concentration of  $5\text{-}15 \times 10^7$  cells/mL. Cells are slowly layered over a 9-volume 20% sucrose cushion and centrifuged at  $480 \times g$  for 5 min. at  $4^{\circ}\text{C}$  to remove cell debris. The pellet is resuspended in 1x PBS and the process is repeated with a second cushion. Cells are resuspended in ice cold 1x PBS + protease inhibitors (50 $\mu\text{g}/\text{mL}$  TLCK, 400 $\mu\text{M}$  PMSF, 10 $\mu\text{g}/\text{mL}$  aprotinin, 16 $\mu\text{g}/\text{mL}$  benzamidine, 10 $\mu\text{g}/\text{mL}$  leupeptin, 2 $\mu\text{g}/\text{mL}$  pepstatin, 10 $\mu\text{g}/\text{mL}$  phenanthroline) to  $2 \times 10^7$  cells/mL. Cells are examined under a microscope to ensure cell debris has been removed. If much cell debris still remains, cells are passed through a third cushion and rechecked for loss of debris. Note: These sucrose cushion conditions are very stringent, i.e. a large percentage of the cells are lost at each step. Centrifuge speed is increased if too many cells are being lost, or decreased if cell debris remains after passing cells through the cushions.

#### *FACS analysis of DNA content*

Samples are processed as above for elutriation fractions. The DNA content is typically  $\sim 100\%$  4N, indicating a 100% G2/M population (Figure 4-4B).

### *Determination of mitotic index by counting condensed chromosomes*

The sample of cells saved for chromosome counting is pelleted by centrifugation for 5 min. at 560 x *g* in a swinging bucket rotor. Cells are resuspended in 100 $\mu$ L 1x PBS and 900 $\mu$ L 10% formaldehyde/90% methanol is added. The sample is incubated at room temp. for 8 min. Fixed cells are pelleted by centrifugation in a swinging bucket rotor for 5 min. at 560 x *g*. Cells are resuspended in 1x PBS to a concentration of  $\sim 2.5 \times 10^7$  cells/mL. 3 $\mu$ L of cells are mounted on a slide with 5 $\mu$ L 80% glycerol and 0.5 $\mu$ L 0.5 mg/mL Hoechst stain and covered with a coverslip.

Cells are counted under a microscope, using the Hoechst stain to distinguish nuclei and condensed chromosomes (Figure 4-4C). The number of cells with condensed chromosomes is tallied and the mitotic index is calculated as: number of cells with condensed chromosomes/total number of cells (Figure 4-4D).

### *Staining procedure*

Cells are kept on ice at all times. All centrifugation is performed at 4°C and all buffers are at 4°C and supplemented with protease inhibitors immediately before use. Fixed and purified colchicine-treated cells are adjusted to a concentration of  $2 \times 10^7$  cells/mL and an equal volume of 0.03% Triton X-100 in 1x PBS is added for a final concentration of 0.016% Triton X-100. Note: Higher concentrations of detergent may give better staining leading to greater separation between FITC-positive and FITC-negative cells, which should allow for a more efficient sort. However higher concentrations of detergent can also lead to more damaged cells



which causes cell clumping, resulting a higher abort rate during the sort. It can also lead to greater adherence of cells to surfaces. Both of these effects decrease the overall recovery of cells.

Cells are incubated on ice for 15 min. It is important that the starting concentration of the cells is accurate, as the cells will be sticky after permeabilization making counting them impossible. Cells are centrifuged at 730 x *g* for 5 min. and washed with 1% BSA, 0.1% Triton X-100 in 1x PBS. Cells are resuspended to a concentration of  $1 \times 10^7$  cells/mL in 1x PBS with FITC-conjugated anti-H3S10p antibody at a concentration of 2.7-3  $\mu\text{g/mL}$ . Notes: 1) Titration of antibody may be necessary to achieve maximal separation of FITC-positive and FITC-negative cells. 2) Non-conjugated antibodies can be used in conjunction with a FITC-conjugated secondary antibody. After the cells are incubated in primary antibody, they are washed with 1% BSA, 0.1% Triton X-100 in 1x PBS and resuspended in 1x PBS with a 1:500 dilution of FITC-conjugated secondary antibody (0.75mg/mL stock) to a final concentration of  $1 \times 10^7$  cells/mL. Cells are incubated on ice for 30 min. in the dark and are washed again with 1% BSA, 0.1% Triton X-100 in 1x PBS.

Cells are incubated in antibody for 30 min. on ice in the dark then centrifuged at 730 x *g* for 5 min. and washed with 1% BSA, 0.1% Triton X-100 in 1x PBS. Cells are resuspended in a volume of horse serum equivalent to one-tenth of the volume required to give  $1.7 \times 10^7$  cells/mL. Nine-tenths volume of 1x PBS is added to give a final concentration of  $1.7 \times 10^7$  cells/mL. Note: This is the concentration at which the

cells will be sorted. The concentration may need to be varied to achieve the most efficient sorting--maximal events/sec with the lowest abort rate.

Cells are incubated overnight at 4°C in the dark. This step greatly improves staining, giving greater separation of FITC-positive and FITC-negative cells, allowing for a more efficient sort.

#### *Pre- and post-sorting procedures*

The cells are very sticky so it is extremely important to coat EVERYTHING that will come into contact with the cells with horse serum. Cells are kept in the dark and on ice when not running through the flow cytometer. Cells are passed through a 40 µm nylon cell strainer and loaded into input tubes in 0.5-1 mL aliquots. Cells are sorted and FITC-positive cells are collected. Gates are set conservatively to obtain the purest population of FITC-positive cells. Cells coming off the flow cytometer are checked on a FACS analyzer to make sure the gating is stringent enough. If the cells are < 95% FITC-positive, gates are reset more conservatively.

Sorted cells are collected in 14mL round bottom glass tubes coated with horse serum, placed on ice. All collected cells are pooled and counted using a hemocytometer. The count should be similar to the counts given by the flow cytometer for the number of particles selected. If the counted number is much less than the number given by the cytometer cells may be sticking to collection tubes, centrifuge tubes or pipet tips. Make sure all surfaces are coated with horse serum prior to bringing them into contact with the cells.

Cells are aliquoted in  $10 \times 10^6$  cell aliquots and centrifuged at  $4^{\circ}\text{C}$  for 5 min. at  $1,920 \times g$  in a spinning bucket rotor. Supernatants are removed and cell pellets are flash frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$ .

Pre- and post-sorted cells are analyzed directly on a FACS analyzer.

Representative results are shown in Figure 4-4E.

#### *Preparation of control cells*

A control population is an asynchronous culture processed in an analogous way as the mitotic cells. Asynchronously growing cells are fixed and stained similarly to the colchicine-treated cells with the following modifications. 1) Sucrose cushions are spun at  $1,920 \times g$  since the asynchronously growing cells are smaller in size. 2) Staining is done with FITC-conjugated anti-histone H3 antibody at a concentration of  $3 \mu\text{g/mL}$ . 3) After permeabilization and washing during the staining procedure 10% of the total cells are separated out and incubated in 1x PBS without antibody. This sample is processed in parallel with the anti-H3 stained cells for the rest of the staining procedure. Before sorting, unstained cells are mixed with the anti-H3 stained cells to give a final composition of 1-2% unstained cells. The unstained cells guide the gating of FITC-positive cells.

#### *Chromatin immunoprecipitation*

Experimental procedures for ChIP are described in Chapter 3.

## References

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## **Chapter 5**

### **Conclusions and Discussion**

Epigenetic mechanisms are responsible for the maintenance of gene expression profiles. How these mechanisms propagate transcriptional states through the process of mitosis is unknown and remains a major outstanding question in epigenetics. The studies presented here address this question by examining how PcG-dependent repression might be propagated through mitosis by systematically characterizing the behavior of PcG proteins during mitosis in *Drosophila* S2 cells. By immunofluorescence and biochemical fractionation PcG proteins were observed to remain associated with mitotic chromosomes. However, by ChIP and ChIP-SEQ PSC and PH are lost from the majority of their interphase binding sites during mitosis. PSC and PH are retained at small subset of their binding sites in mitosis, which include sites at domain borders. These data suggest a model in which persistent binding sites may nucleate re-binding of PcG proteins to target sites upon mitotic exit. The implications of these results, remaining questions and future directions are discussed below.

The data presented in Chapter 2 provides evidence that a substantial fraction of all PcG proteins tested fractionated with mitotic chromosomes. The fraction of chromatin-associated PSC in mitotic cells was 51% of the fraction of chromatin associated PSC in control cells, and the same number for PH is 86% (Table 2-1). Yet data presented in Chapter 3 showed that PSC and PH are bound to about 12% and 3% of their interphase sites in mitosis, respectively, and on average, binding to these sites was reduced (Figure 3-2). This leads to the question as to the nature of the protein that fractionates with mitotic chromatin yet does not seem to bind to target

sequences. It is possible a technical explanation could be found, i.e. the protein is bound at target sites in mitosis but is not accessible by ChIP. Alternatively, this protein may be loosely associated with the chromatin. The continued association of PcG proteins during mitosis, even if non-specific, may assure equal segregation of the proteins to daughter cells or may keep the concentration of PcG proteins near chromatin high to allow rapid reformation of functional complexes and chromatin structures upon mitotic exit. Additional fractionation combined with salt extraction may provide further insight into the nature of this association. However it seems unlikely that memory of the transcriptional status of specific target genes resides in this population of the protein.

ChIP on G2/M cells suggests the other PRC1 components PC and dR also are lost from PREs in the BX-C (Figure 3-7). Loss of SU(Z)12 and PHO from these sites may indicate that full PRC2 and PhoRC complexes are also lost from these sites. It is possible that E(Z), ESC, SFMBT are retained at PREs in the BX-C and facilitate reformation of PRC2 and PhoRC at these loci, as they were not tested here. It is clear, however that persistent binding of PSC and PH, and likely PC, dR, PHO and SU(Z)12 do not contribute to the maintenance of transcriptional memory through mitosis by remaining bound to PREs in the BX-C.

Two features examined here were retained at the BX-C in mitosis. H3K27me3 is retained at PREs and PSC is retained at domain borders flanking the BX-C. It is formally possible that either one alone, both or neither are responsible for the

maintenance of repression through mitosis, and each of these scenarios will be examined in turn.

Can H3K27me3 alone be sufficient for directing the rebinding of PcG complexes upon mitotic exit? As discussed in Chapters 1 and 3 a purely targeting role for H3K27me3 seems unlikely given the broad distribution of H3K27me3, the discrete binding pattern of most PcG proteins and the depletion of H3K27me3 at PREs. In this study the binding of H3K27me3 was only examined at PREs in the BX-C, so it is possible that the broad distribution of this modification is lost during mitosis and it is retained only at PREs, although this scenario seems unlikely. The loss of H3K27me3 can occur through three avenues: demethylation by the demethylase dUTX (Smith et al., 2008), dilution of H3K27me3-containing nucleosomes during replication or through histone replacement. dUTX colocalizes with the elongating form of RNA pol II (Smith et al., 2008), and is thus correlated with active transcription. At most a 2-fold dilution might be expected with dilution of H3K27me3 marks through replication, and PREs and domain borders are sites of histone turnover not gene bodies (Deal et al., 2010; Mito et al., 2007), thus a mechanism for the loss of H3K27me3 across its broad domains can not be easily envisioned.

Can PSC retained at domain borders be sufficient for directing the rebinding of PcG complexes upon mitotic exit? Perhaps. The reformation of higher order contacts and reorganization of chromatin into domains upon mitotic exit, mediated by mechanisms that drive higher order folding of the genome, may allow the re-



binding of PcG to binding sites within each domain, nucleated by proteins bound at the border sites and driven by intradomain contacts. The presence of active genes within PcG domains may present a challenge to this model where PcG proteins indiscriminately bind to sites within each domain as a consequence intradomain contacts. How might PcG proteins bound at domain borders lead to the discrimination between active and repressed genes? It is possible that PSC at border sites work in conjunction with H3K27me3, as per the model presented in Chapter 3. H3K27me3 has been shown to cover the coding regions of repressed genes, but not active ones (Beisel et al., 2007; Schwartz et al., 2006; Schwartz et al., 2010), thus may allow discrimination between active and repressed genes. Alternatively, the discrimination of active vs. repressed may not lie at the level of PcG protein binding. Many studies have described the binding of PcG proteins to genes in both the active and the repressed state (Beisel et al., 2007; Kwong et al., 2008; Papp and Müller, 2006; Ringrose et al., 2004; Schuettengruber et al., 2009). Thus PcG binding may be re-established over the whole domain upon mitotic exit and a separate mechanism, such as retention of PcG complexes by H3K27me3, may be used to distinguish the active vs. repressed state.

Finally it is possible that neither H3K27me3 nor persistent binding of PSC at domain borders are required for propagation of the repressed state during mitosis. The functional requirement for either of these persistent features specifically during mitosis remains to be demonstrated. A dearth of methods available for reversible mitotic arrest in *Drosophila* cells limits the experimental options through which this

hypothesis may be tested. If such methods were available, mitotic arrest in combination with RNAi or targeted degradation of PcG proteins may allow the removal of PcG proteins during mitosis. Similarly the inducible overexpression of H3K27me3 demethylases may allow the demethylation of H3K27me3 during mitosis. The expression of PcG target genes, or a fluorescent reporter controlled by a PRE, could be monitored during the subsequent G1 to assess the requirement for either PcG proteins or H3K27me3 during mitosis for target gene repression.

The high degree of colocalization of mitotic PSC and PH sites with the chromatin insulator proteins BEAF, CTCF, CP190 and the protein Chromator warrants further investigation. All four proteins were also shown to overlap with domain borders (Sexton et al., 2012), and BEAF, CTCF and CP190 along with GAF and the insulator protein Mod(mdg4) were previously found to bind the boundaries of H3K27me3 domains (Bartkuhn et al., 2009; Nègre et al., 2010). As BEAF and CP190 were found to associate with mitotic chromosomes (Hart et al., 1999; Whitfield et al., 1995), it will be important to determine whether these insulator proteins also bind to these sites in mitotic chromosomes. Perhaps there is an interdependence of binding between PcG proteins and insulator proteins at these mitotic sites. Indeed, preliminary evidence suggests Mod(mdg4) exist in a mitosis-enriched complex with PSC (A. Wani, personal communication).

CTCF, Mod(mdg4) and CP190 are also bound to PREs in the BX-C during interphase (Mohan et al., 2007; Nègre et al., 2010). If these proteins remain bound to PREs during mitosis they also may play a role in the re-binding of PcG proteins

upon mitotic exit, as insulator proteins are thought to mediate long range interactions both in the BX-C (Ferraiuolo et al., 2010; Li et al., 2011) and elsewhere in the genome (reviewed in Phillips and Corces, 2009; Wallace and Felsenfeld, 2007; Yang and Corces, 2012). It was shown that the histone H3 and H3K27me3 depletion observed at H3K27me3 domain borders (domain borders are sites of high nucleosome turnover (Deal et al., 2010; Mito et al., 2007)) is lost in *cp190* and *ctcf* mutant larvae, suggesting a role for these proteins in either nucleosome depletion or turnover (Bartkuhn et al., 2009). As the data presented in Chapter 3 gives preliminary evidence for H3K27me3 and H3 depletion/high turnover at PREs in the BX-C during mitosis, perhaps CTCF and CP190 or another factor involved in creation of such chromatin structure remains, which may in turn facilitate the rebinding of PcG complexes upon mitotic exit.

The implication of both PcG proteins and insulator proteins in the formation of higher order structures, coupled with the presence of each at domain borders, and the persistence of PSC at those borders during mitosis, may indicate that higher order chromatin structure may be involved in carrying transcriptional memory. While preliminary evidence suggests that higher order chromatin structures within the BX-C are reduced in G2/M cells (C. Lanzuolo and V. Orlando, personal communication) persistence of both insulator proteins (which has yet to be demonstrated) and PcG proteins at domain border sites (Chapter 3) in mitosis may be involved in reforming higher order chromatin structures upon mitotic exit. To the extent that higher order chromatin structures are causally linked to transcriptional

activation or repression, reformation of higher order structures may be important for transcriptional memory (reviewed in Deng and Blobel, 2010).

In this work mitosis was considered as a single event, while it is actually a dynamic process consisting of several distinct steps and a multitude of players that change as mitosis progresses. It is possible that one mechanism may not be sufficient to explain the maintenance of repression by PcG proteins through mitosis. Different marks, structures or proteins may have varying importance during the different stages of mitosis, and sequential interactions with the changing landscape of mitotic structures and factors may serve to “hand-off” the memory of repression through the process. Indeed different behaviors with respect to chromatin binding was observed for PSC vs. PC and PH at the various stages of mitosis (Buchenau et al., 1998).

The studies presented here suggest a possible role for chromatin organization in epigenetic memory through mitosis. The extent to which remnants of higher order structure that persist in mitosis affects the reformation of such structures and associated transcriptional states during the subsequent G1 remains to be determined. Such a mechanism may be important for the propagation of PcG-dependent repression through mitosis and may even play a more general role in mediating epigenetic memory through mitosis.

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## **Appendix**

### **Polycomb Proteins Remain Bound to Chromatin and DNA during DNA Replication *In vitro***

The work presented in this Appendix has been published.

Francis, N. J., Follmer, N. E., Simon, M. D., Aghia, G., Butler, J. D., 2009. Polycomb proteins remain bound to chromatin and DNA during DNA replication *in vitro*. *Cell*. 137, 110-122.



## Summary

The transcriptional status of a gene can be maintained through multiple rounds of cell division during development. This epigenetic effect is believed to reflect heritable changes in chromatin folding and histone modifications or variants at target genes, but little is known about how these chromatin features are inherited through cell division. A particular challenge for maintaining transcription states is DNA replication, which disrupts or dilutes chromatin-associated proteins and histone modifications. PRC1-class Polycomb group protein complexes are essential for development and are thought to heritably silence transcription by altering chromatin folding and histone modifications. It is not known whether these complexes and their effects are maintained during DNA replication or subsequently re-established. We find that when PRC1-class Polycomb complex-bound chromatin or DNA is replicated *in vitro*, Polycomb complexes remain bound to replicated templates. Retention of Polycomb proteins through DNA replication may contribute to maintenance of transcriptional silencing through cell division.

## Introduction

The hypothesis that chromatin structure is the basis for many epigenetic phenomena implies that it can be propagated through cell division. Transfer of chromatin-based gene regulatory information through DNA replication would require that specific chromatin features (chromatin folding, chromatin-associated proteins, histone variants, and histone modifications) are inherited or recreated with

fidelity (Ng and Gurdon, 2008; Nightingale et al., 2006). Pioneering *in vitro* studies demonstrated that histones remain associated with DNA through replication, even in the absence of cellular factors (reviewed in Annunziato, 2005; Bonne-Andrea et al., 1990). *In vivo*, parental histones and at least some histone modifications are inherited by daughter chromatin during DNA replication, although the mechanistic details of this process are not known (Annunziato, 2005; Benson et al., 2006). Extensive data demonstrating that regulatory proteins, including histone-modifying complexes themselves, can recognize specific histone modifications have led to a model for propagation of gene regulatory information through cell division. In this model, modified histones are transferred from parent to daughter DNA, where they recruit enzyme complexes that propagate the same modifications to newly added histones (Dodd et al., 2007; Ng and Gurdon, 2008; Nightingale et al., 2006). However, there is no direct evidence yet for this model in the context of DNA replication, and the fate of most nonhistone chromatin-associated epigenetic factors during DNA replication has not been determined.

*Drosophila* Polycomb group (PcG) proteins maintain transcriptional silencing and are believed to act through epigenetic mechanisms (Grimaud et al., 2006). The best understood PcG target genes are the homeotic (Hox) genes, which control segmental identities during development. Transcriptional repression of Hox genes in specific segments early in development is initially established by transiently expressed transcription factors but is subsequently maintained by PcG proteins. The pattern of Hox gene repression is stable through multiple rounds of cell division

despite the decay of the early-acting transcription factors (Ringrose and Paro, 2004; Simon and Tamkun, 2002).

Recent work in *Drosophila* and mammals implicates PcG proteins in biological functions beyond stable regulation of Hox genes. Genome-wide studies of PcG protein binding have identified many new target genes and implicated PcG proteins in a wide range of developmental processes (Kwong et al., 2008; reviewed in Pietersen and van Lohuizen, 2008; Ringrose, 2007; Schwartz and Pirrotta, 2008). Mechanistically, these studies indicate that PcG regulation can be dynamic according to cell type and differentiation stage. PcG proteins also direct a range of gene expression levels not restricted to silencing. Early studies of Polycomb response elements (PREs), the DNA recognition elements that target PcG proteins in *Drosophila*, indicated that PcG proteins maintain silencing established early in development but do not silence genes that are active early in development (Poux et al., 2001; Ringrose and Paro, 2004). These data suggest that the PcG system can recognize the transcriptional status of a gene. Consistent with this, more recent studies suggest that PcG proteins can be targeted to genes that are poised for activation and that PcG proteins play a central role in ES cell pluripotency and differentiation by maintaining this potentiated state (Kwong et al., 2008; Pietersen and van Lohuizen, 2008).

Biochemical studies suggest that PcG proteins function in multiprotein complexes. Polycomb repressive complex 1 (PRC1) includes four core PcG subunits: polyhomeotic (Ph), posterior sex combs (PSC), dRING, and Polycomb (Pc) (Francis et

al., 2001; Lavigne et al., 2004; Levine et al., 2002; Saurin et al., 2001; Shao et al., 1999). A reconstituted complex of these four proteins or three of the four can alter chromatin and DNA structure, inhibit chromatin remodeling, and inhibit transcription of both naked DNA and chromatin (Francis et al., 2004; Francis et al., 2001; King et al., 2002; Mohd-Sarip et al., 2006; Shao et al., 1999). Components of PRC1 can also function as an E3 ligase for histone H2A ubiquitylation (Cao et al., 2005; de Napoles et al., 2004; Elderkin et al., 2007; Wang et al., 2004a) probably in the context of a distinct complex (Lagarou et al., 2008). All of these activities correlate with silencing *in vivo* (Cao et al., 2005; Cao and Zhang, 2004; Grimaud et al., 2006; King et al., 2005; Lagarou et al., 2008; Ringrose and Paro, 2004; Wang et al., 2004a).

A second PcG complex, PRC2, whose components are also essential for gene silencing, can methylate histone H3 on lysine 27 (H3K27me). A component of PRC1, Pc, can recognize H3K27me3 via its chromodomain (Fischle et al., 2003; Min et al., 2003). It is hypothesized that PRC2 is recruited to PREs, where it methylates surrounding nucleosomes; methylation of H3K27 leads to recruitment of PRC1 and silencing (Cao and Zhang, 2004; Wang et al., 2004b). Similarly, if H3K27me3 is transferred to daughter DNA during replication, it could recruit PRC1-class complexes if they are disrupted by passage of the DNA replication fork. This model is supported by the presence of high levels of H3K27me3 around PREs and the loss of binding of Pc at PREs when expression or function of E(Z), the catalytic subunit of PRC2, is reduced (Cao and Zhang, 2004; Wang et al., 2004b). Recent work in

mammalian cells indicates that PRC2 may also recognize H3K27me3, suggesting a mechanism for propagation of the methylation mark (Hansen et al., 2008).

PRC1 may also function independently of PRC2 and H3K27me3 in some cases. PHO, a protein that binds to many PREs (Brown et al., 1998; Klymenko et al., 2006; Mihaly et al., 1998) can directly contact and recruit a recombinant PRC1-class complex *in vitro* (Mohd-Sarip et al., 2005). Some PcG binding sites lose PRC1 components when PRC2 components are reduced, but others do not (Wang et al., 2004b), and H3K27me3 does not always colocalize with PcG binding (Ringrose et al., 2004). In mouse embryos, PRC1 is targeted to heterochromatin in the paternal genome independent of PRC2 and H3K27me3 (Puschendorf et al., 2008) and to the imprinted locus *Kcnq1* independent of *Ezh2* (Terranova et al., 2008).

The focus on H3K27me3 and histone modification in general as the principle means by which chromatin may transmit epigenetic memory through replication implicitly assumes that chromatin proteins other than histones dissociate during the biochemical process of DNA replication. To test this idea, we examined the effect of DNA replication on the association of PRC1-class PcG protein complexes with chromatin and naked DNA *in vitro*.

## Results

### *Inhibition of Chromatin Remodeling by PCC Is Preserved through Chromatin*

#### *Replication In vitro*

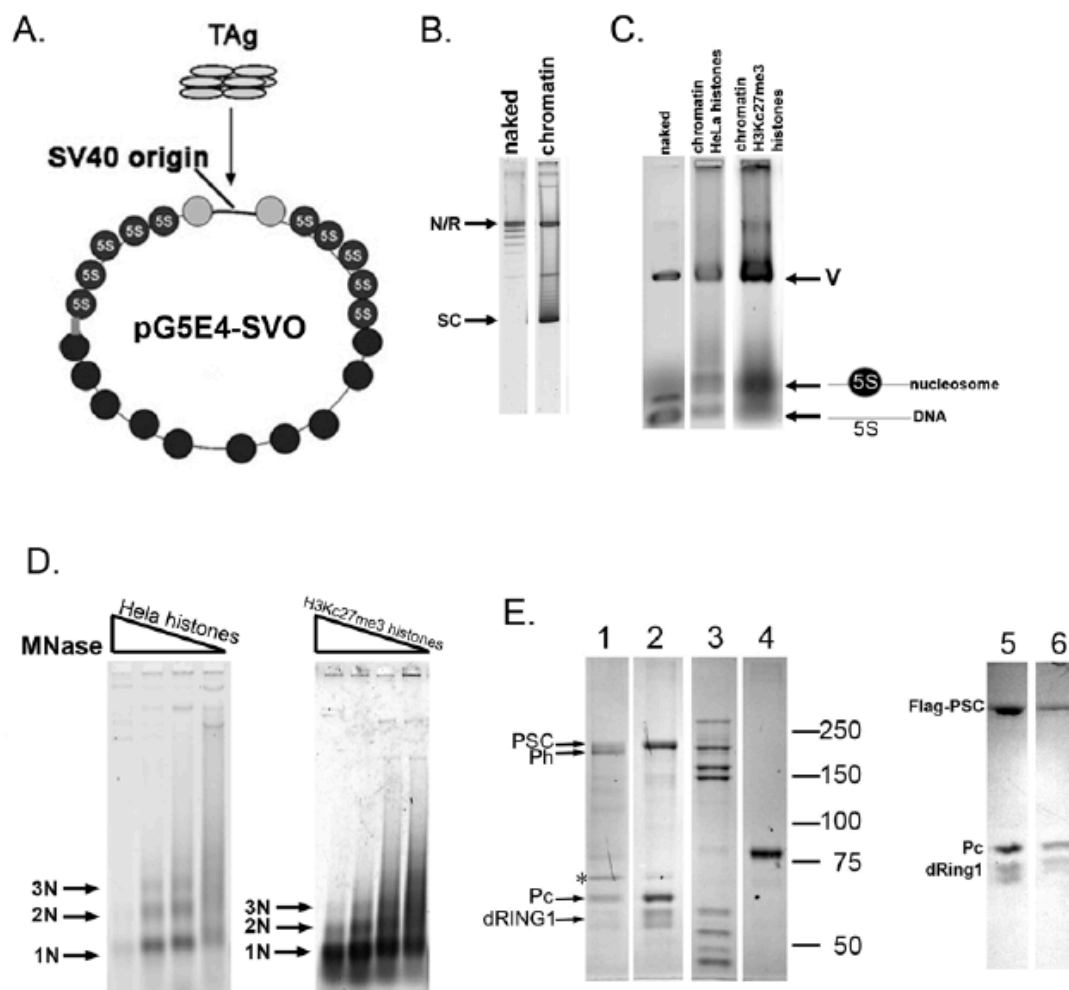
To test the effect of DNA replication on PRC1 core complex (PCC)-bound chromatin, we used the well characterized cell-free Simian Virus 40 (SV40) DNA replication system (Li and Kelly, 1984; Stillman and Gluzman, 1985). The SV40 protein large T-Antigen (TAG) binds specifically to the replication origin of SV40 viral DNA or to plasmids containing this sequence to initiate bidirectional DNA replication. TAG and PCC were sequentially bound to chromatin templates containing the SV40 origin, and S100 cytoplasmic extracts were added to initiate DNA replication (templates and proteins are described in Figure S1 available online). DNA replication was monitored by  $\alpha^{32}\text{P}$ -dATP incorporation (Figure 1A).

An aliquot of each replication reaction was used in a restriction enzyme accessibility assay (Francis et al., 2001; Logie and Peterson, 1997; Polach and Widom, 1995). In this assay, chromatin remodeling by hSwi/Snf increases restriction enzyme accessibility, and this effect is blocked by PCC (Francis et al., 2001). All of the experiments reported here were carried out with *Drosophila* PCC containing PSC, dRING, and Pc; in some experiments, Ph was included (PCC + Ph). Although the original studies with PCC were carried out with the four-protein complex, in our previous work and in these studies, complexes with and without Ph behaved identically (Francis et al., 2004; Francis et al., 2001; Lavigne et al., 2004).

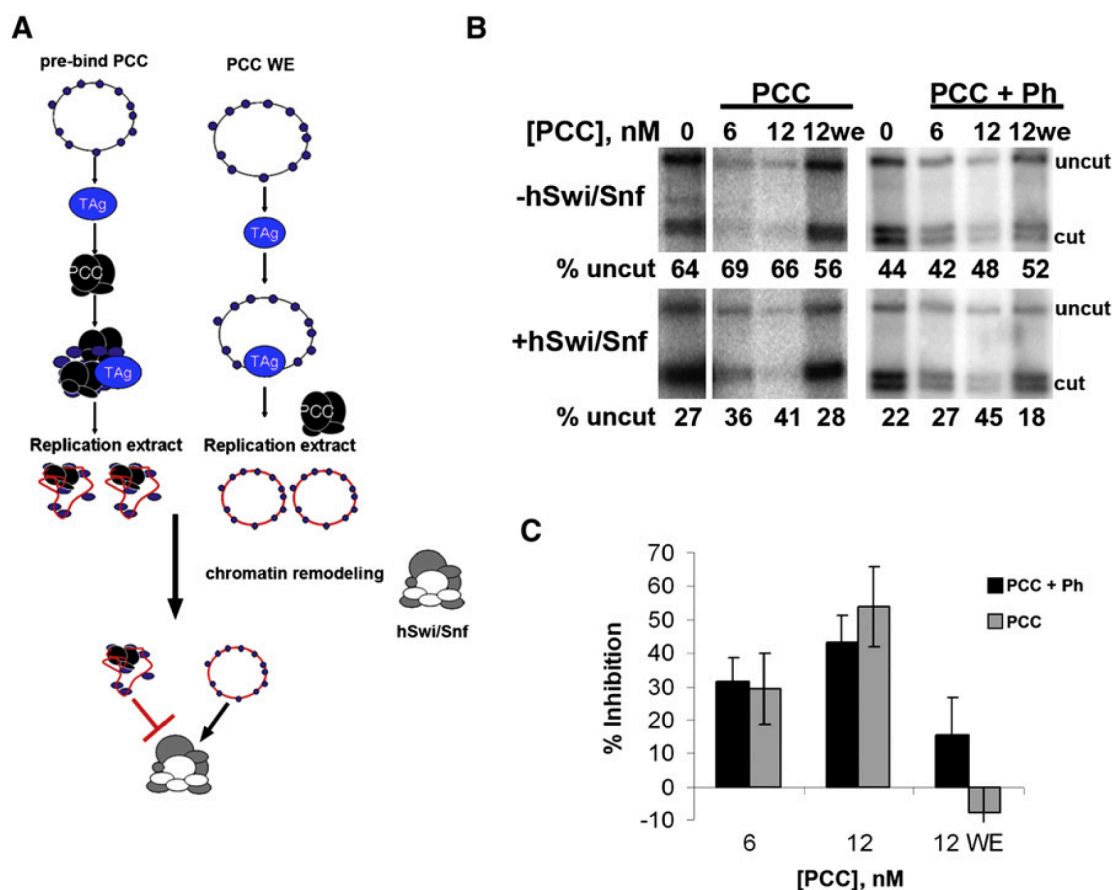
When PCC was bound to chromatin before replication, less chromatin remodeling occurred on replicated templates than in reactions without PCC (Figures 1B and 1C). This effect is dose dependent and occurs over a similar range, as previously described for inhibition of chromatin remodeling (partial inhibition is

**Figure S1. Templates and proteins used in these studies.** **A.** Schematic diagram of template used for chromatin replication. This template is modified from pG5E4 (Utley et al., 1998) and has the SV40 origin of replication (from the SVO+ plasmid, Stillman and Gluzman, 1985), between two sets of 5 nucleosome positioning sequences (5S). **B.** Supercoiling gel of chromatin templates demonstrates that templates are assembled into nucleosomes. 100 ng of naked DNA or chromatin were treated with Topoisomerase I (5U, 60 min. at 37°C), digested in stop solution, deproteinated, run on a 1.3% agarose, 1X TAE gel, and visualized by staining with SYBR gold. Arrows point to the migration position of nicked (N) and relaxed (R) DNA and supercoiled (SC) DNA. **C.** Native agarose gel of EcoRI digested chromatin templates. EcoRI cuts between the 5S nucleosome positioning sequences, releasing nucleosome sized fragments (Carruthers et al., 1999); when these fragments are bound by nucleosomes, they migrate more slowly through the agarose gel. 300 ng of template were digested with EcoRI, and DNA was visualized by ethidium bromide staining. The level of assembly over the 5S region was determined by comparing the nucleosome occupied (slow migrating) and unoccupied EcoRI fragments V=vector backbone which lacks EcoRI sites. The H3Kc27me3 histone templates are used in Figure 6. **D.** Micrococcal nuclease (MNase) analysis of chromatin template used for replication demonstrates nucleosome assembly. 2 µl Micrococcal nuclease (MNase) (diluted in 50 mM Tris, pH 8, 10 mM NaCl, 126 mM CaCl<sub>2</sub>, 5% glycerol) were added to 10 µl of chromatin at 2.5 ng/µl in 12 mM Hepes, pH 7.9, 0.24 mM EDTA, 12% glycerol, 60 mM KCl. Digestions were allowed to proceed for five minutes at room temperature and stopped with DSB-PK. DNA was separated on 1.3% agarose 1X TAE gels and visualized by SYBR gold staining. 1N, 2N, 3N indicate the positions of mono, di and trinucleosomes. We note that templates used for chromatin replication are undersaturated with nucleosomes, evident both in the heterogeneous distribution of topoisomers in **B** and in the amount of unbound EcoRI fragments in **C**. **E.** Gelcode Blue (Pierce) stained SDS-PAGE of proteins used in these experiments. Note that all proteins were purified from baculovirus infected Sf9 insect cells except for hSwi/Snf (lane 3). Lane 1: PCC (Francis et al., 2001); Lane 2: PCC without Ph. Note that as observed in other assays, PCC behaved identically whether or not Ph was included (Francis et al., 2004; Lavigne et al., 2004); Lane 3: hSwi/Snf purified from HeLa cells expressing Flag-tagged Ini-1 (Sif et al., 1998); Lane 4: SV40 Large T-Antigen (Landford, 1988); Lane 5 PCC; Lane 6 biotin-labeled PCC.

Figure S1 (Continued)







**Figure 1. PCC Inhibits Chromatin Remodeling of Replicated Templates**

(A) Reaction scheme for *in vitro* replication followed by chromatin remodeling. The scheme on the right is the control in which PCC is added with the replication extract (WE). (B) Example of restriction enzyme accessibility assay carried out after *in vitro* replication of chromatin. ATP-dependent chromatin remodeling by hSwi/Snf decreases the fraction of the template that is uncut; this reaction is inhibited when PCC is bound to chromatin prior to *in vitro* replication. Panel shows phosphorimager scan of chromatin remodeling reactions so that only replicated templates (that incorporated  $\alpha^{32}\text{P}$ -dATP during replication) are visible. PCC is composed of PSC, Pc, dRING; PCC + Ph also contains Ph. (C) Summary of inhibition of chromatin remodeling of replicated templates. Error bars in all figures represent the SEM unless otherwise indicated.

observed at one complex for 3–5 nucleosomes, or approximately five PCCs per plasmid). In these experiments, both PCC and nucleosomes are likely above their  $K_D$  for interaction, and nucleosomes are in excess of PCC, but PCC is in excess of plasmids (see Figure S2 for further explanation). When PCC is added with the replication extracts instead of prebound to chromatin (indicated as “WE” in figures), inhibition of chromatin remodeling is not observed, suggesting that binding of PCC to chromatin is prevented in extracts. Thus, chromatin templates bound to PCC prior to DNA replication behave as though PCC is bound after DNA replication in that they are refractory to chromatin remodeling.

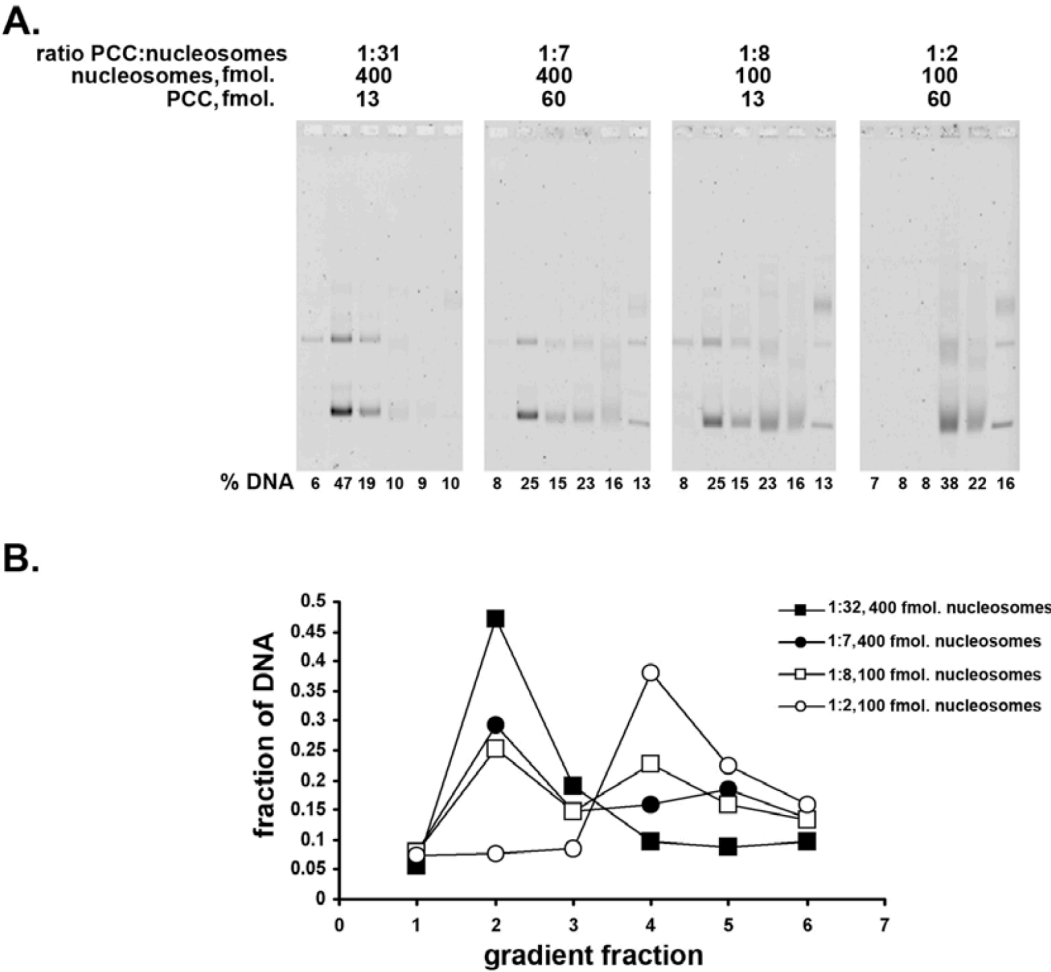
#### *PCC-Bound Templates Are Completely Replicated*

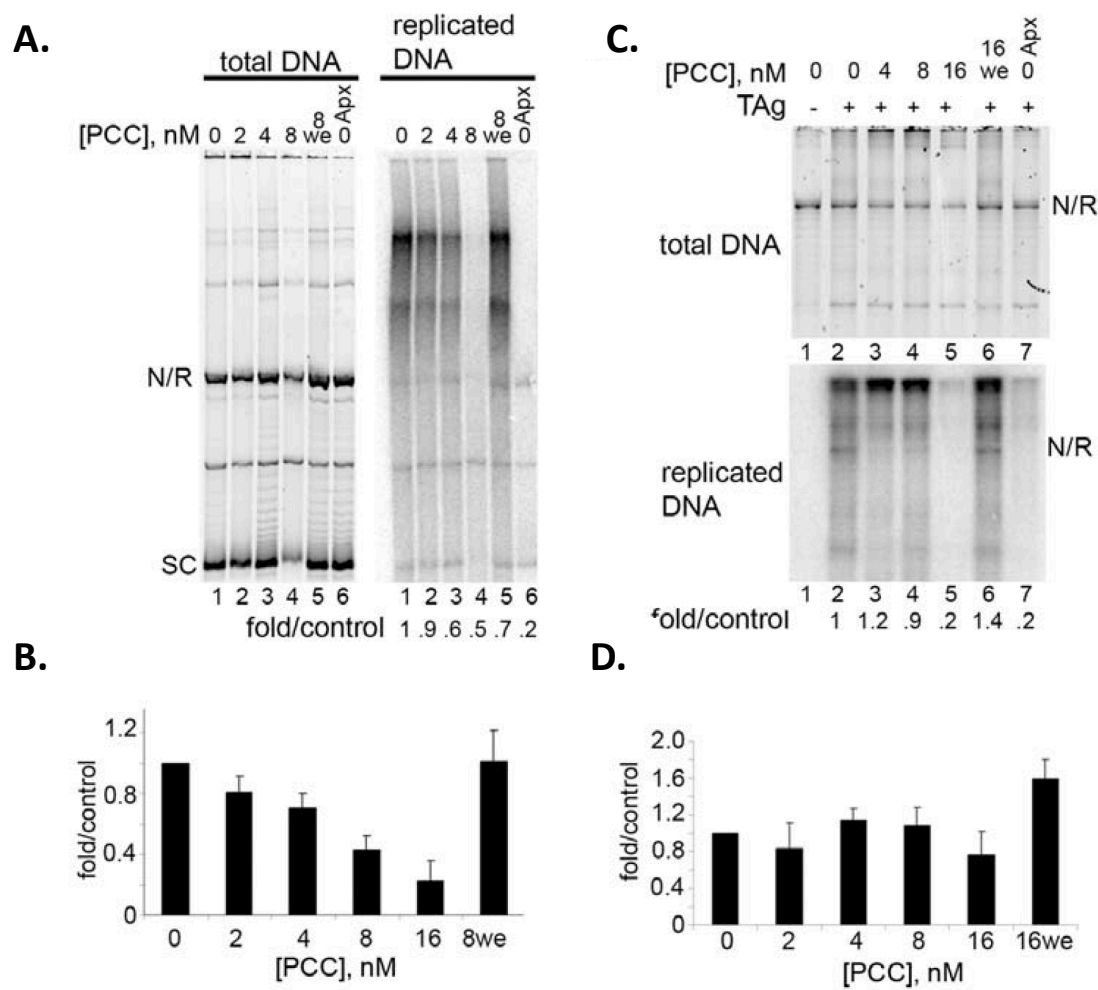
Our results suggest that PCC, or its effect on chromatin, is preserved through DNA replication. Alternatively, PCC-bound templates may be partially replicated and PCC bound to unreplicated segments. This possibility was of particular concern because PCC inhibits replication in a dose-dependent manner (Figure S3). If PCC blocks passage of DNA replication forks, then PCC-bound templates should generate partially replicated products, but if PCC inhibits replication initiation, then fewer products should be generated in reactions with PCC bound to the template, but they should be full length. We used three assays to determine whether full-length replication products are produced from PCC-bound templates (Figure 2). Under our replication conditions, chromatin replicates about three times less efficiently than naked DNA. To increase chromatin replication, we used a fraction from *Xenopus*

**Figure S2. Both PCC and template are likely above  $K_D$  in under standard reaction conditions.**

**A.** Previous characterization of PCC demonstrated that it inhibits nucleosome remodeling (which presumably reflects chromatin binding) at  $\sim 1$  nM, and the  $K_D$  of PCC for a 155bp DNA fragment is 0.2 nM (Francis et al., 2001). If both PCC and plasmid templates are above their  $K_D$  in our reactions, and the amount of binding observed should reflect the ratio between PCC and nucleosomes. We compared binding of PCC to template at 1.25 and 6 nM of PCC complex and 10 or 40 nM nucleosomes (0.5 or 2 nM plasmid) using sucrose gradient sedimentation. Following binding, reactions were separated on sucrose gradients, and panels show native agarose gels to demonstrate the position of the template in the gradient. Similar binding is observed for 1.25 nM PCC with 10 nM nucleosomes (0.5 nM plasmid) as for 6 nM PCC with 40 nM nucleosomes (2 nM plasmid); in these reactions the ratio of PCC:nucleosomes is the same. When 6 nM PCC is used with 10 nM nucleosomes (0.5 nM plasmid), more binding is observed than with 40 nM (2 nM plasmid). In this experiment, if the template concentration was below  $K_D$ , the fraction of template bound should stay the same; instead we observe increased binding when the concentration of template is dropped but the ratio of PCC to nucleosomes is increased. Note that in later experiments, full binding sufficient to shift the plasmid occurs at a ratio of about 1 complex per 5 nucleosomes, which is consistent with our previous finding that one complex can compact a 4-nucleosome array (Francis et al., 2004). Given that the nucleosomal templates we use for DNA replication are slightly underassembled (typically 0.7- 0.9 histone:DNA), it is likely that the ratio of PCC:nucleosomes is closer to 1:4. These data also imply that about 5 PCCs are bound to each plasmid. **B.** Quantification of binding from **A.** These results are consistent with the template being above the  $K_D$  for binding since decreasing template concentration increases binding and allows lower concentrations of PCC to shift the template.

Figure S2 (Continued)





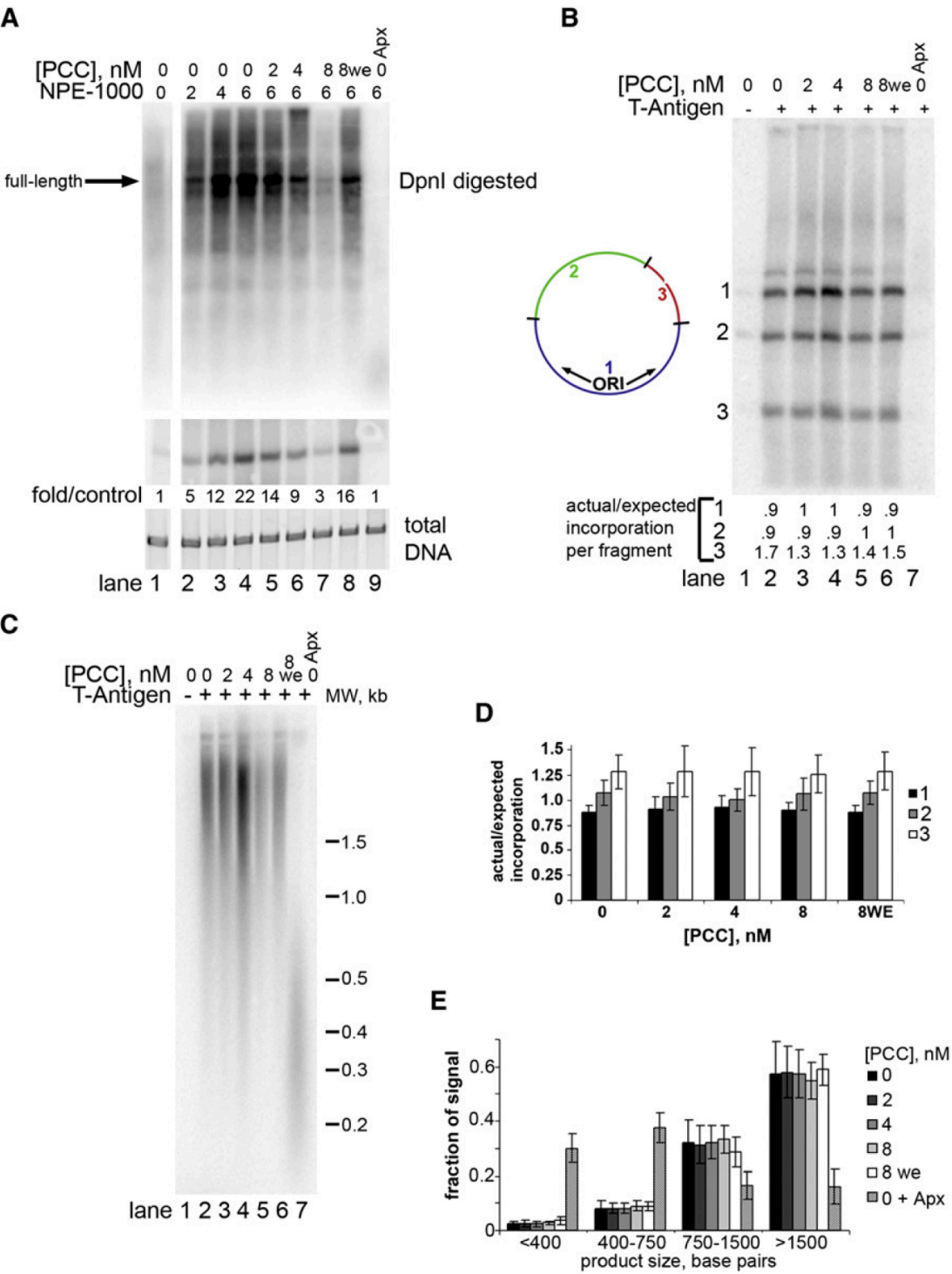
**Figure S3. Inhibition of replication by PCC on chromatinized or naked DNA templates.**

**A.** Native agarose gels of chromatin replicated with the indicated amount of PCC. Left panel shows total DNA visualized by SYBR gold and the right panel is phosphorimager scan of incorporated radioactivity. SC marks the position of supercoiled and N/R the position of nicked or relaxed templates. **B.** Summary of the effect of PCC on DNA replication. **C, D** are the same as **A, B** except that naked DNA was used as the template and panels in **C** are total DNA (top) and phosphorimager scan (bottom). Note that the template migrates at the position of relaxed plasmid in the top panel of **C**, confirming that chromatin assembly is not occurring on naked DNA templates. All error bars in figures are SEM unless otherwise indicated.

**Figure 2. Full-Length Replication Products Are Present after Replication of PCC-Bound Chromatin and DNA**

(A) Top panel is phosphorimager scan of chromatin replication reactions digested with DpnI. Middle panel is a phosphorimager scan of the same reaction products linearized with a restriction enzyme, and bottom panel shows total DNA (SYBR gold stain). NPE-1000 was added to stimulate replication (see Figures S4 and S5) (B) Phosphorimager scan of replication products digested with three restriction enzymes as indicated. Replication should initiate in fragment 1. The ratio of actual/expected incorporation in each fragment is indicated below each lane. (C) Denaturing gel of replication products to show length of nascent strand. (D) Graph summarizing the actual/expected incorporation into each DNA fragment from experiments like the one in (B). (E) Graph summarizing the distribution of radioactivity in replication products analyzed by denaturing gel electrophoresis as in (C). Each lane was divided into four segments, and signals are expressed as a fraction of the total signal for the lane. Aphidicolin (Apx) was added after 25 min (lane 9 in [A], 7 in [B] and [C]) to stall replication forks.

Figure 2 (Continued)

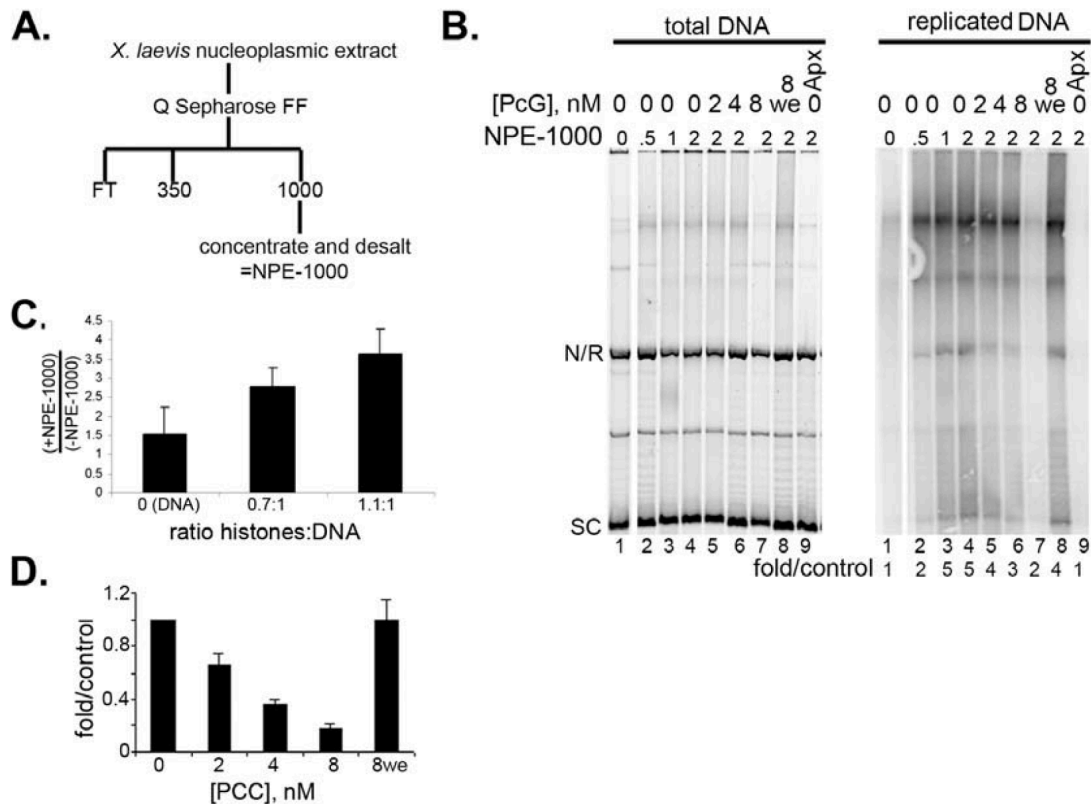


extracts that contains high levels of the histone chaperone nucleoplasmin (referred to as NPE-1000) (Figures S4 and S5) in some of our experiments.

The restriction enzyme DpnI can distinguish replicated from unreplicated DNA. DpnI digests DNA that is adenine-methylated in its recognition site (GA'TC). Plasmids used in these experiments were propagated in bacteria and therefore are fully methylated at their 26 DpnI sites. Hemimethylated DNA in which one strand is methylated and the other is not is digested 60-fold more slowly than methylated DNA (Sanchez et al., 1992). Replicated DNA is hemimethylated and DpnI resistant. DpnI-resistant, full-length products were observed (Figures 2A and S6A) whether or not PCC was prebound to the chromatin and from reactions carried out on naked DNA templates (Figure S6D).

As a second test for full-length replication products, replicated plasmids were digested into three fragments, one of which contains the SV40 origin of replication. If replication initiates but fails to complete, then incorporation of  $\alpha^{32}\text{P}$ -dATP should occur preferentially near the origin (fragment 1 in Figure 2B). The ratio of actual to expected incorporated  $\alpha^{32}\text{P}$ -dATP was close to 1 for each fragment for both naked DNA and chromatin, implying that the entire plasmid was replicated (Figures 2B, 2D, S6B, S6C, S6E, and S6F). Fragments 2 and 3 were consistently overrepresented in reactions from chromatinized but not naked DNA templates (compare Figure 2D with S6F). We do not understand this result, but note that the ratio of incorporation into the three fragments is not altered by PCC. As a third test for partial replication products, denaturing gels were used to separate the nascent and template DNA





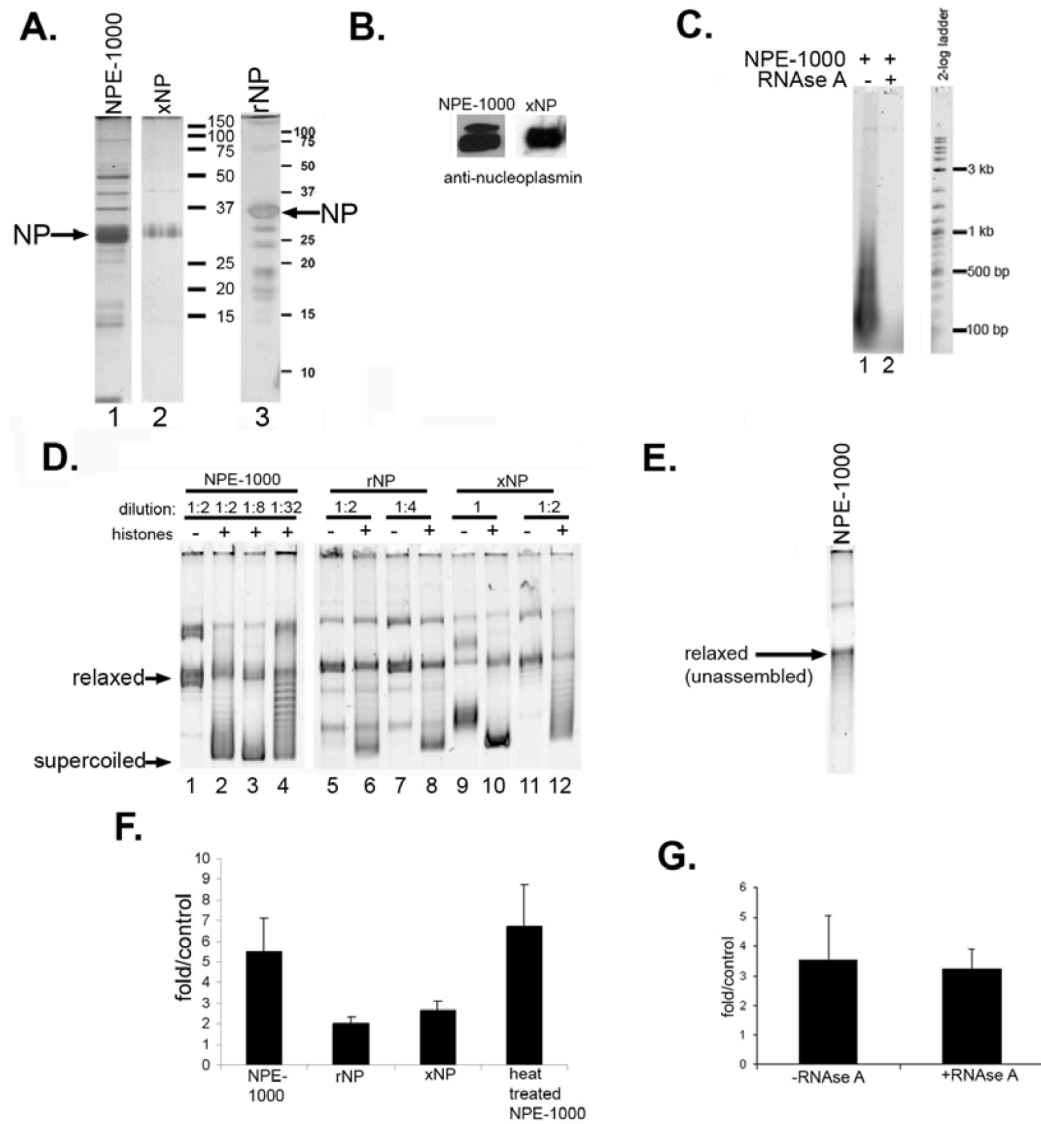
**Figure S4. *Xenopus laevis* nucleoplasmic extract fraction stimulates replication of chromatin in the presence and absence of PCC.**

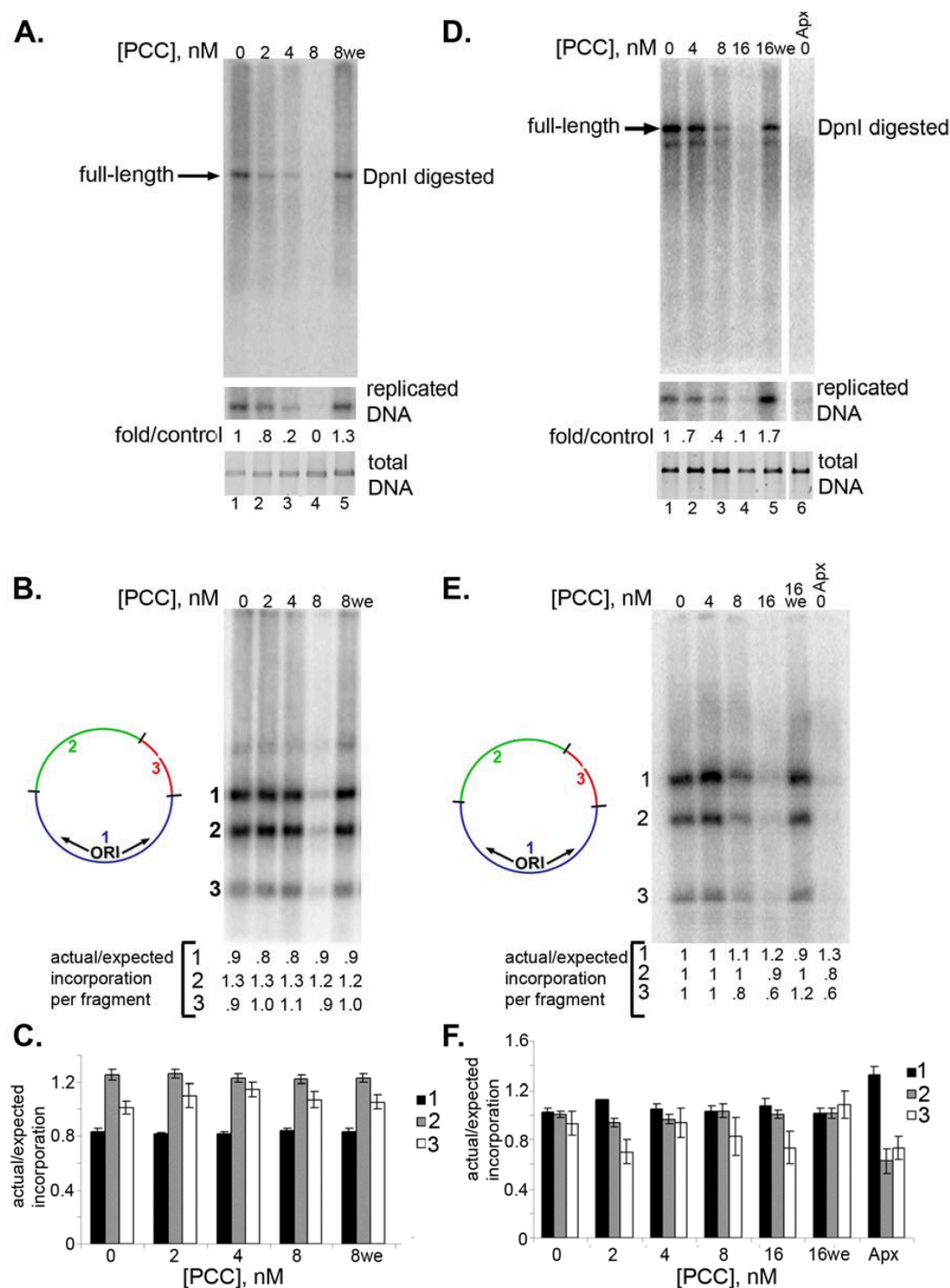
**A.** Nucleoplasmic extract (NPE) was prepared from *Xenopus laevis* eggs (Walter et al., 1998) and fractionated as indicated. **B.** A high salt elution from an anion exchange column, which we refer to as NPE-1000, (about 1.5  $\mu$ g of protein per 25 ng of chromatin template) stimulates Tagdependent, Aphidicolin sensitive (Apx, lane 9) replication of chromatin by at least 3 fold. NPE-1000 increases replication but does not affect inhibition of replication by PCC. Left panel shows total DNA by SYBR gold staining, and right panel is a phosphorimager scan of incorporated  $^{32}$ P-dATP. NPE-1000 does not alter supercoiling of either total or replicated templates suggesting it is not assembling or removing nucleosomes. SC marks the position of supercoiled and N/R the position of nicked or relaxed templates. **C.** Summary of the effect of NPE-1000 on replication of naked DNA and chromatin indicating that NPE-1000 does not stimulate replication of naked DNA under these conditions. **D.** Summary of the effect of PCC on replication of chromatin in the presence of NPE-1000, demonstrating that inhibition occurs over the same range as for chromatin in the absence of NPE-1000 (compare with sFig. 3B).

**Figure S5. Nucleoplasmin may be the active component in NPE-1000 that stimulates replication of chromatin.**

**A.** Silver stained gel of 2.5 $\mu$ l NPE-1000, 10  $\mu$ l nucleoplasmin purified from *Xenopus* extracts (xNP, purified according to (Sealy et al., 1989)) (lane 2), and 1 $\mu$ l NP from *E. coli* (rNP, gift of W.M. Michael, Harvard University) (lane 3); arrows point to nucleoplasmin which is the major protein component in the fraction. **B.** Western blot confirming the presence of nucleoplasmin in NPE-1000 and xNP. **C.** SYBR gold stained agarose gels of NPE-1000 demonstrating that it contains RNA. 10  $\mu$ l of NPE-1000 were treated with buffer or RNase A for 15 min. at 37 °C, digested with Proteinase K and separated on a 1.3% agarose 1X TAE gel. **D.** NPE-1000, xNP, and rNP have nucleosome assembly activity. Protein fractions were incubated with HeLa histones (125 ng) for 20 minutes at room temperature followed by the addition of 75 ng of DNA and Topoisomerase I. Reactions were incubated 90 minutes at 30 °C, digested with Proteinase K, separated by agarose gel electrophoresis and stained with SYBR gold. Neither rNP or xNP are as active as NPE-1000. However, rNP lacks the phosphorylation present on NP in *Xenopus* egg extracts that is implicated in NP function (Cotten et al., 1986; Sealy et al., 1986) and xNP is about 8-fold less concentrated than NP in NPE-1000. **E.** NPE-1000 does not assemble chromatin in replication extracts. NPE-1000 was incubated with S100 relaxed extract to see if it can serve as a source of histones. SYBR gold stained gel demonstrates that plasmids remain relaxed (unassembled) under these conditions. **F.** rNP, xNP, and NPE-1000 heated to 80 °C all stimulate replication of chromatin. NP is an usual protein in that it is heat stable (Laskey et al., 1978), so the insensitivity of NPE-1000 to heating is consistent with NP being the active component in it. **G.** RNaseA treated NPE-1000 stimulates replication of chromatin. Error bars in **F** and **G** are standard deviations.

Figure S5 (Continued)





**Figure S6. Full-length replication products can be produced from chromatin and naked DNA in the absence of NPE-1000 .**

These experiments are similar to Fig. 2 except that no NPE-1000 was added. **A.** Phosphorimager scan of DpnI digested (top panel) and linearized reaction products (middle). Bottom panel is a SYBR gold stain of total DNA. **B.** Triple digest of replication products indicates that incorporation into all three fragments is similar. **C.** Summary of incorporation of radioactivity into each fragment during *in vitro* DNA replication. **D-F** are the same as **A-C** except that naked DNA is the template.

strands (Figures 2C and 2E). Large products were observed in both the presence and absence of PCC (Figure 2C, lanes 3–6), but when aphidicolin was added to stall DNA polymerase, products less than 500 base pairs accumulate (Figure 2C, lane 7; Figure 2E). Some partial replication products are detected in these assays, particularly from chromatin templates, but they are not increased when PCC is added. We conclude that PCC effects on chromatin persist through DNA replication *in vitro*.

#### *PCC Remains Bound to Replicated Chromatin and DNA*

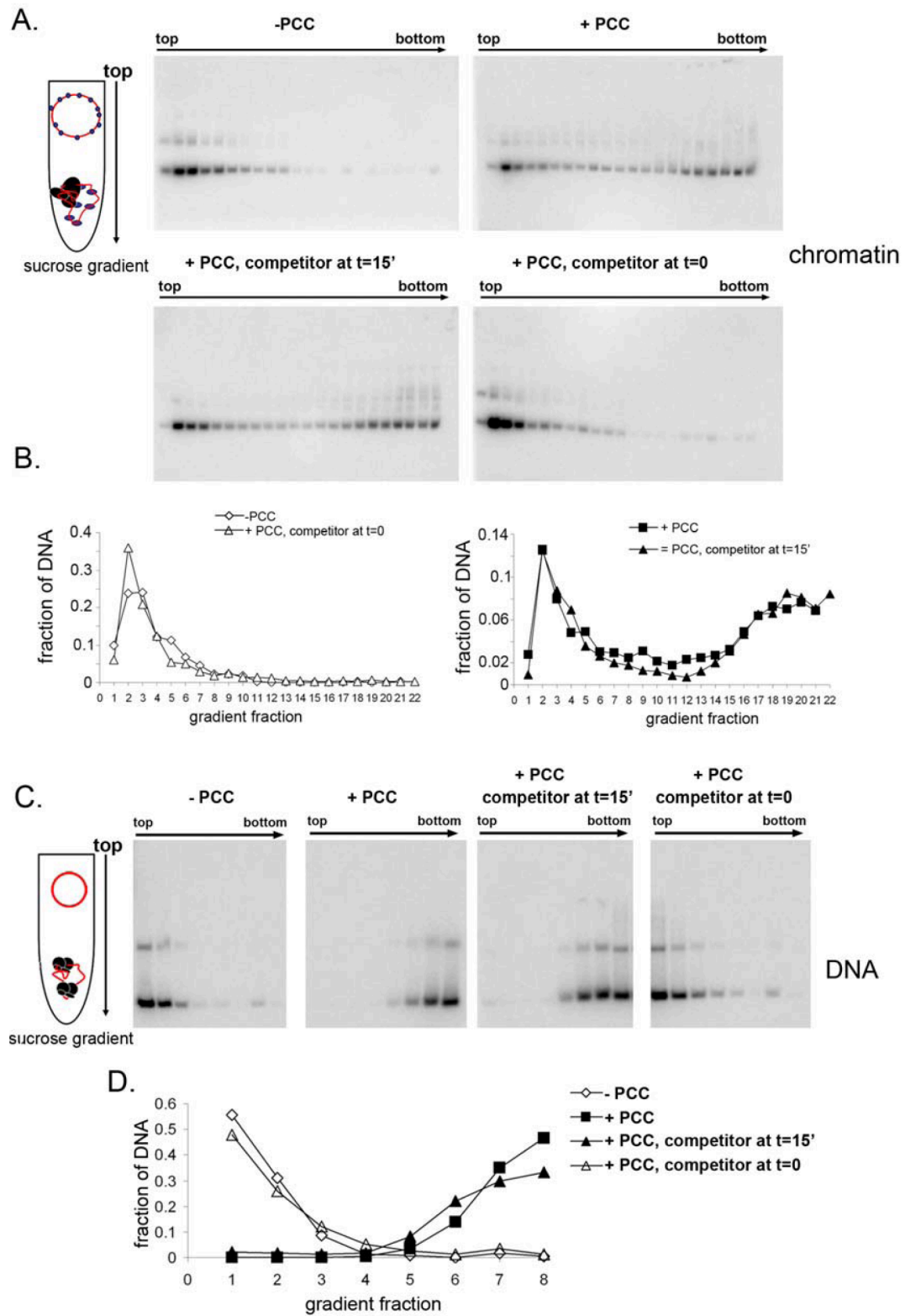
To test whether PCC is bound to replicated templates, we first characterized binding of PCC to DNA and chromatin by sucrose gradient sedimentation. Chromatinized plasmids bound to PCC sediment further into sucrose gradients than unbound ones (Figures S7A and S7B). Binding was not disrupted by competitor chromatin, indicating that it is stable under these conditions. A 2-fold higher concentration of PCC was required to shift naked DNA than chromatin in the gradient, but DNA binding was also stable to competitor challenge (Figures S7C and S7D). These results suggest that once PCC is bound to a template, it remains bound over the time scale relevant for *in vitro* DNA replication.

To determine whether replication dissociates PCC from chromatin, we separated PCC-bound and unbound templates by sucrose gradient sedimentation after DNA replication *in vitro* (Figure 3). Fractions were analyzed by agarose gel electrophoresis; total DNA was analyzed by SYBR gold staining, and replicated DNA

**Figure S7. Stable binding of PCC to chromatin and DNA.**

**A.** To determine whether PCC binding to chromatin is stable, PCC was incubated with 50ng of chromatin template at a ratio of 1 complex: 5 nucleosomes (~5 complexes per plasmid) (sFig. 7). After allowing the complexes to bind, 500ng of a second chromatinized plasmid were added (t=15 min.). As a control, the competitor was added during the initial binding step (t=0). In this case, PCC should associate with the competitor, which is in excess of both template and PCC. The reactions were incubated for 90 minutes at 30°C, and then fractionated by sucrose gradient sedimentation. Fractions were analyzed on agarose gels, transferred to nylon membranes and probed with an oligonucleotide probe that recognizes the template but not the competitor. **B.** Graphs of quantification of the amount of template in each fraction. Left graph shows reactions –PCC or with competitor added at t=0 while right graph shows +PCC or +PCC with competitor at t=15 minutes reactions. **C, D** are the same as **A, B** except that both the template and competitor are naked DNA rather than chromatin. We note that for all of the experiments presented here, nucleosomes are not required but lower concentrations of PCC are needed for effects on chromatinized than naked DNA templates. This could be explained if PCC binds to the free DNA between nucleosomes; however, our previous electron microscopy work of PCC binding to sparsely assembled nucleosomal templates suggests that even when few nucleosomes are present, PCC preferentially interacts with them since we observe nucleosome clustering and free loops of DNA (Francis et al., 2004). We note that a similar relationship between naked DNA and chromatin was observed for PCC inhibition of transcription (King et al., 2002). Note that the gradient conditions in these assays are not directly comparable to those in Figures 3 or sFigure 11 which likely explains the difference in migration pattern.

Figure S7 (Continued)

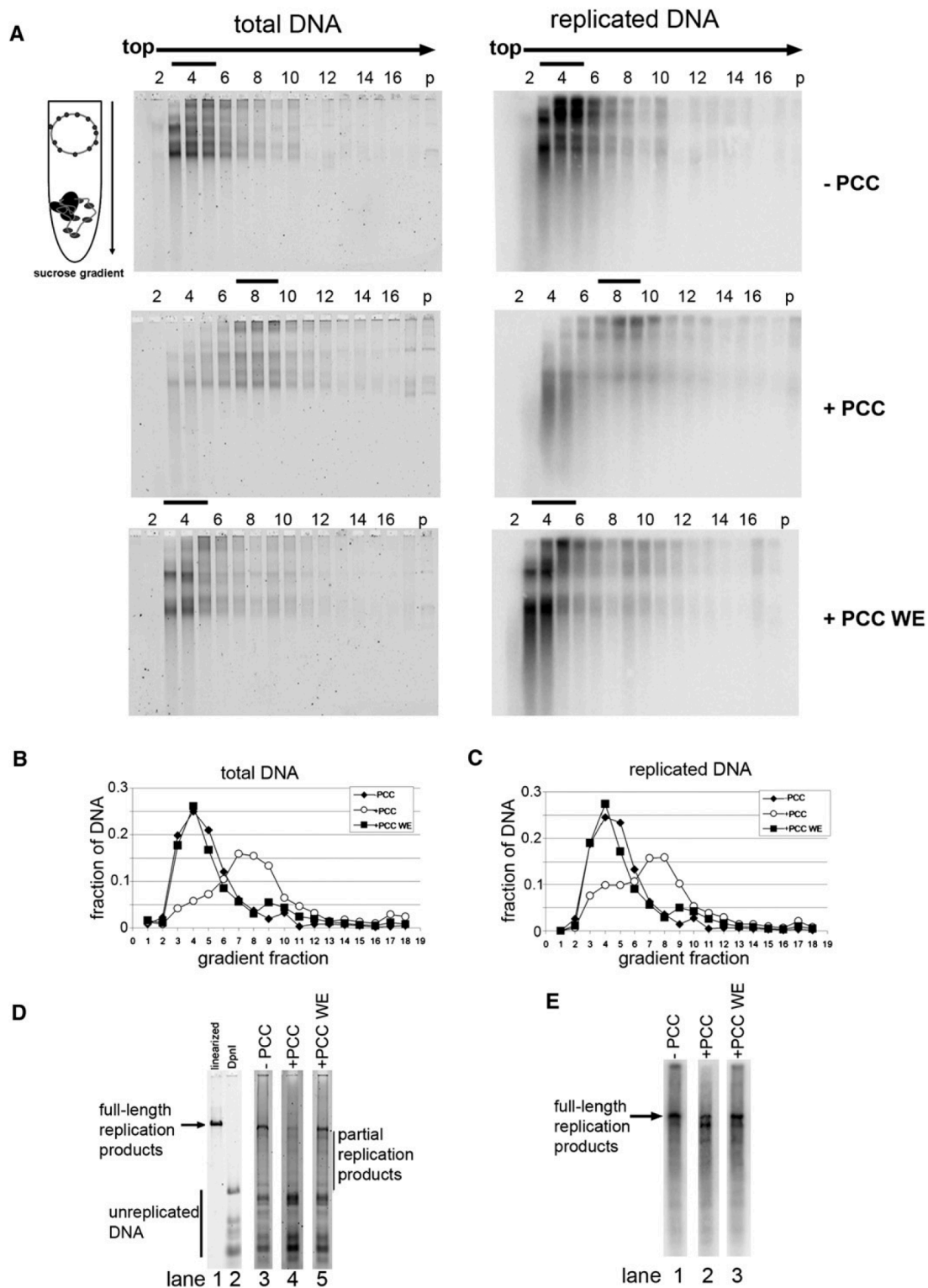


**Figure 3. Association of PCC with Chromatin after Replication In Vitro**

(A) Agarose gels of sucrose gradient fractions from chromatin replication reactions. The left panel shows total DNA (SYBR gold), and the right panels replicated DNA (phosphorimager scan). (B and C) Quantification of gels shown in (A). (D) Fractions were pooled as indicated by black bars in (A), and DNA was purified and digested with DpnI digestion; the panel shows SYBR gold stained agarose gel. Lane 1 shows linearized template and lane 2 completely DpnI digested template. (E) Phosphorimager scan of the reactions shown in (D). Note that lane 2 is a longer exposure than 1 or 3 in (D) and (E).



**Figure 3 (Continued)**



by  $\alpha^{32}\text{P}$ -dATP incorporation. When PCC was bound to chromatin before addition of replication extracts, both replicated and total chromatin sedimented further in the gradient than unbound chromatin (Figures 3A–3C). PCC also cosediments with chromatin templates (Figure S8B). Little evidence for release of PCC from chromatin (which would cause more of the radiolabeled DNA to sediment in upper fractions) was observed. In contrast, when PCC is added with the replication extract, both replicated and unreplicated chromatin sediment like unbound chromatin (Figures 3A–3C).

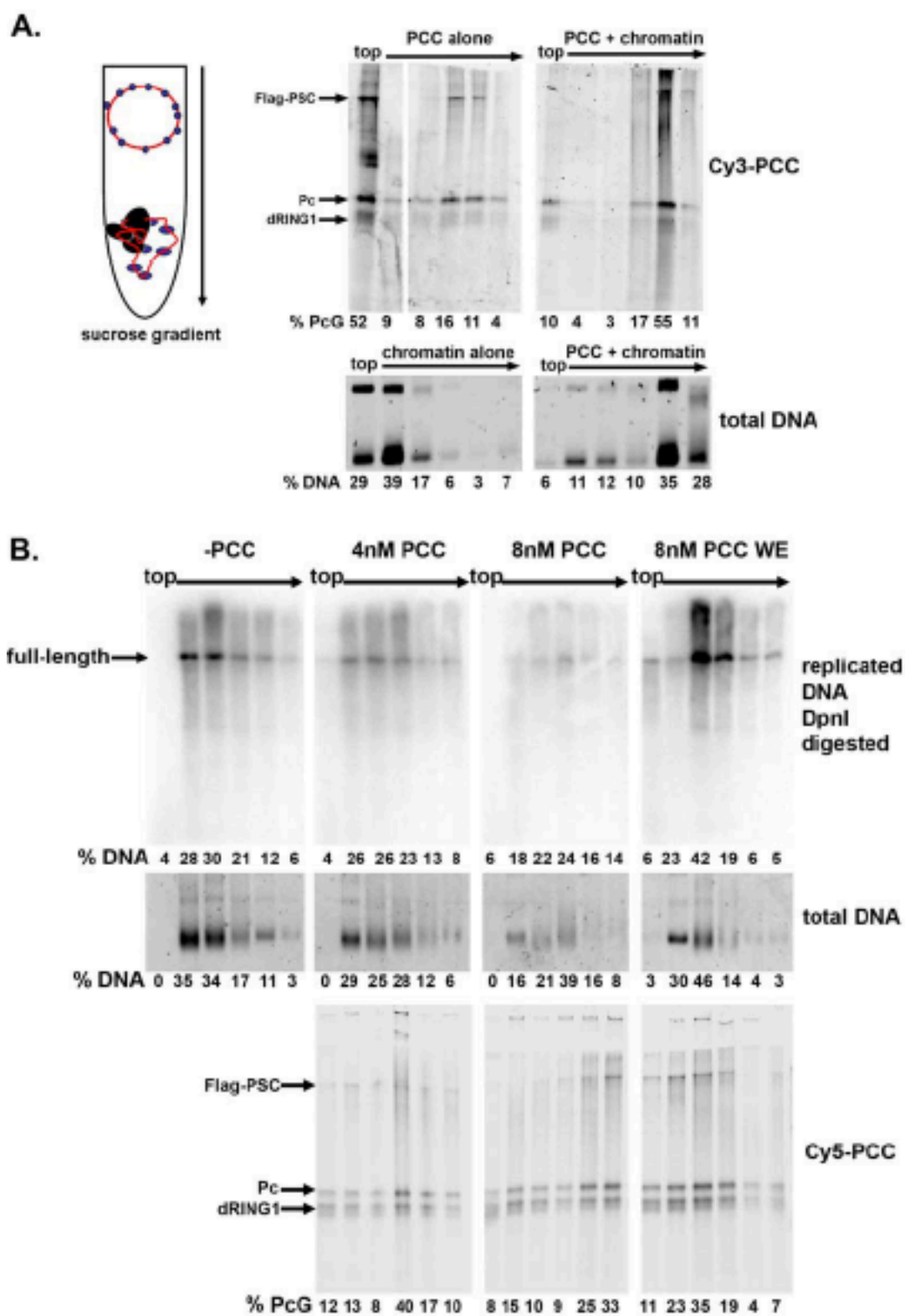
For confirmation that that PCC-bound templates were completely replicated and peak gradient fractions were pooled and analyzed with DpnI. Full-length replication products were observed in fractions containing putative PCC-bound chromatin (lane 4 in Figure 3D and lane 2 in Figure 3E). These observations were confirmed with mini sucrose gradients (Figures S9A and S9B).

Replication reactions using naked DNA as a template were also fractionated by sucrose gradient sedimentation, and again full-length, DpnI-resistant replication products were observed in peak fractions irrespective of PCC addition (Figures S9C, S9D, and S10). In the experiments with naked DNA, we sometimes observed a small peak of replicated DNA sedimenting in the position of unbound templates, suggesting that PCC is released from some templates during replication.

#### *PCC Can Be Crosslinked to Replicated Templates*

**Figure S8. Analysis of PCC complex sedimentation through sucrose gradients.**  
**A.** Protein (top panel) and DNA (bottom panel) gels of gradient fractions from reactions containing PCC alone (left protein gel), chromatin alone (left DNA gel), or PCC and chromatin (right protein and DNA gels). **B.** Replication reactions were separated on sucrose gradients and fractions were analyzed for DNA content (middle panel), full length replication products (top panel, phosphorimager scan of DpnI digested replication products), and PCC (bottom panel). Note that some of the PCC sediments rapidly through the gradients even in the absence of chromatin.

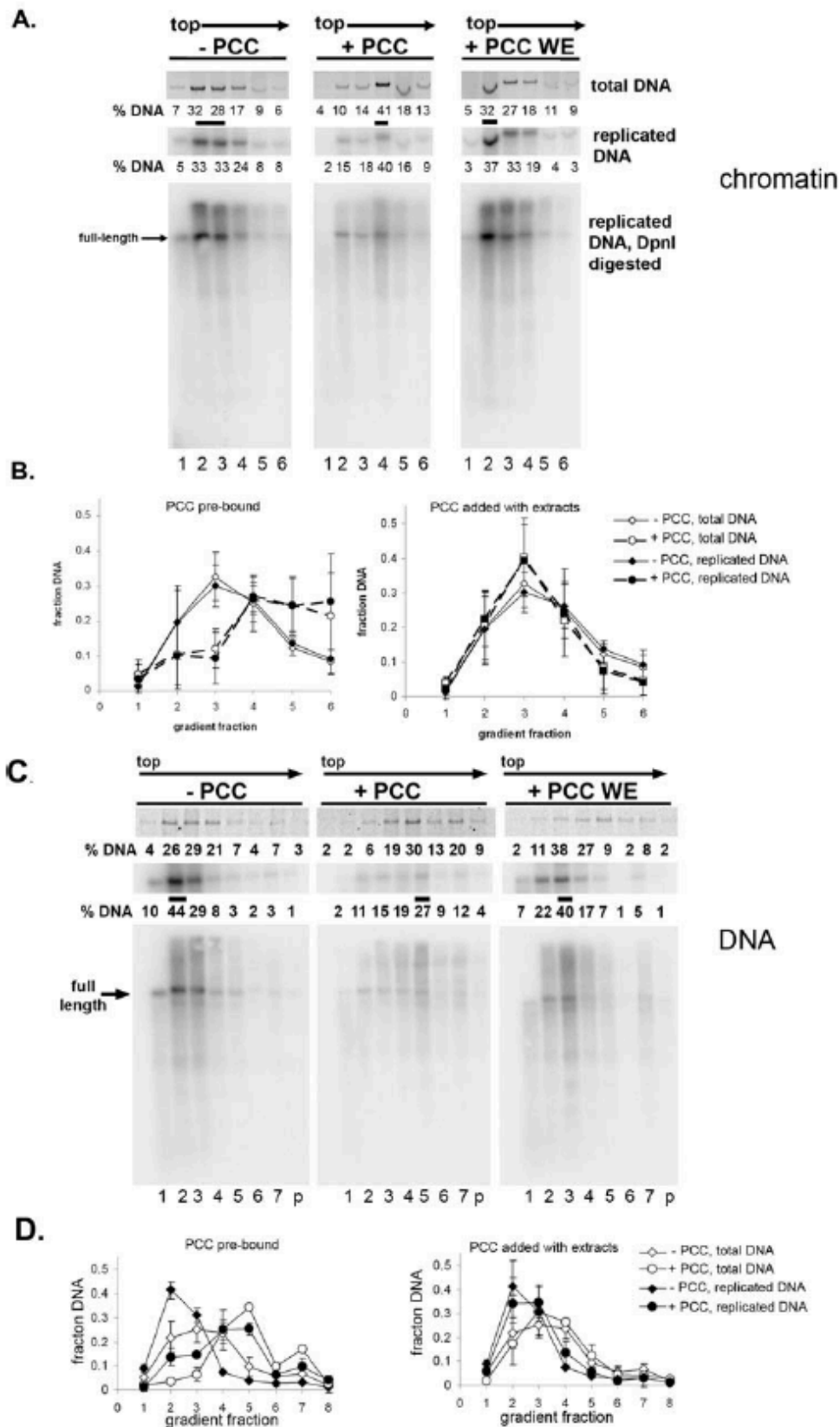
Figure S8 (Continued)

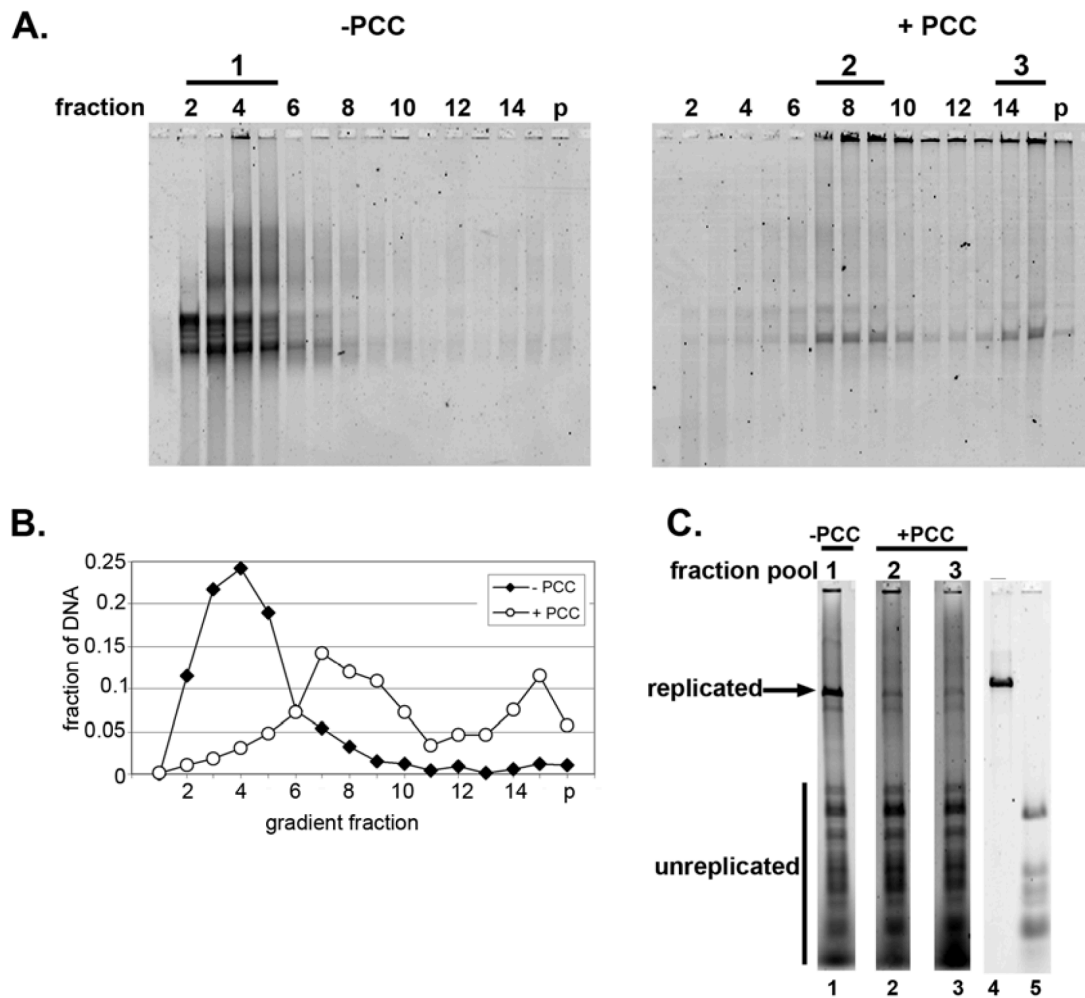


**Figure S9. Separation of replication reactions using chromatin or naked DNA as a template on mini sucrose gradients.**

**A.** Replicated chromatin sediments with PCC bound chromatin through a sucrose gradient. Reactions in which PCC were added before or after replication extracts were fractionated on mini sucrose gradients. Top panel shows linearized total DNA (stained with SYBR gold) and middle panel replicated (radioactive) DNA. The peak fraction in each case is indicated with a black bar. Bottom panel shows that replication products are largely DpnI resistant. **B.** Summaries of sucrose gradient fractionation of replication reactions. Left panel compares templates not incubated with PCC to those pre-bound by PCC, while right panel compares reactions without PCC to those in which PCC are added with the replication extracts. **C** and **D** are similar to **A** and **B** except that the template is naked DNA rather than chromatin. “p” refers to the pellet.

Figure S9 (Continued)





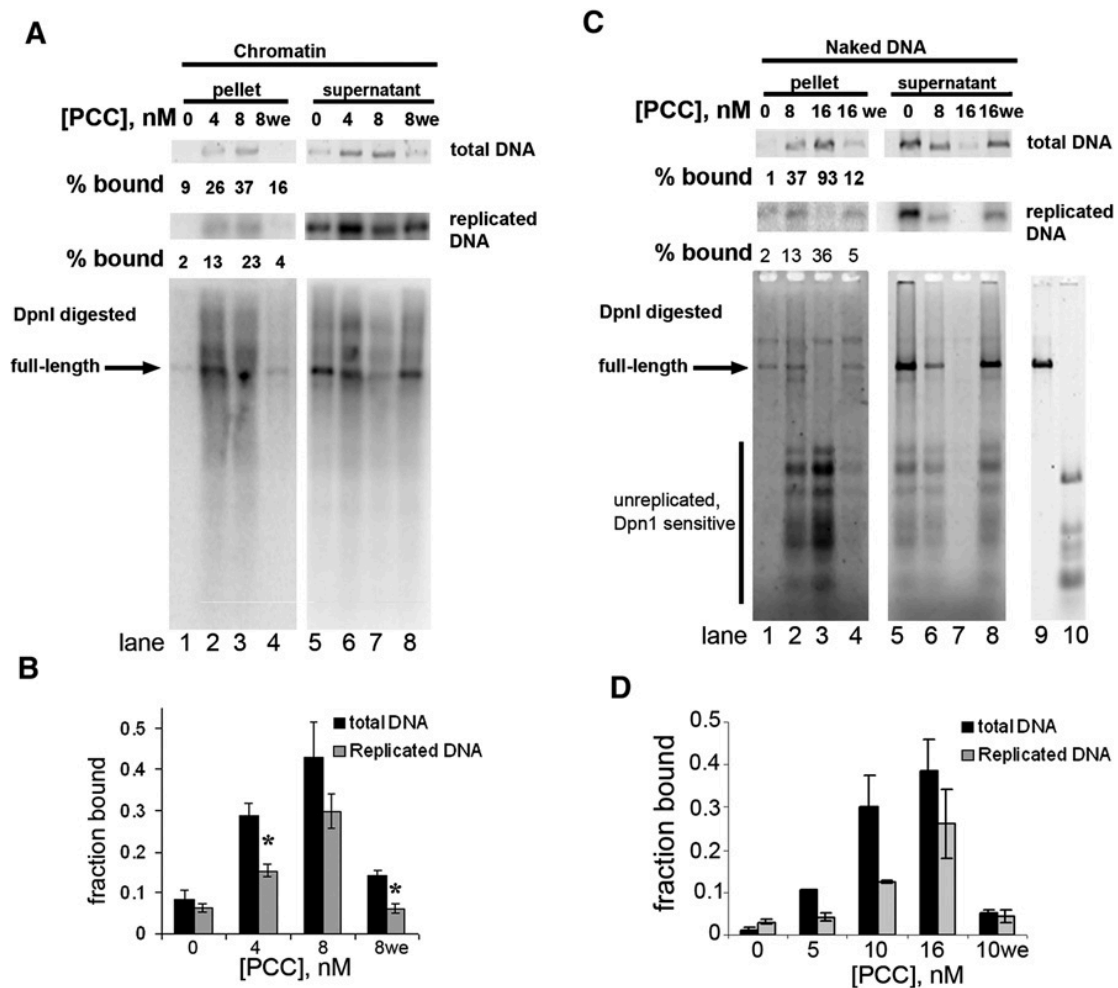
**Figure S10. Association of PCC with naked DNA following replication *in vitro*.**

**A.** SYBR gold stained gels of fractions from sucrose gradients, indicating that DNA incubated with PCC migrates more rapidly through gradients than DNA alone. Fractions were pooled as indicated by black bars and DNA was isolated for DpnI analysis of replication. **B.** Quantification of gels shown in **A**. **C.** SYBR gold stained agarose gel of DNA from indicated fraction pools that was digested with DpnI. Lane 4 shows linearized template which should migrate similarly to replicated templates, and lane 5 shows DpnI digested template which should migrate similarly to unreplicated templates.

As a second test for PCC binding to chromatin after DNA replication, we used biotinylated PCC to precipitate PCC-bound templates (Figure S1E). Replication reactions were crosslinked with formaldehyde, and biotinylated PCC-bound chromatin was recovered with streptavidin-coated beads. A substantial fraction of both total and replicated chromatin was retained on the beads when templates were prebound with PCC (Figures 4A and 4B), implying that PCC remains bound to both replicated and unreplicated chromatin. A greater fraction of total than replicated chromatin was retained on the beads, suggesting that PCC can dissociate during replication (Figure 4B). This difference is significant for low but not high concentrations of PCC, and may be overestimated since templates with fewer PCC bound are more likely to replicate but less likely to be captured by streptavidin-coated beads. Only a fraction of PCC is captured under our conditions, which likely explains capture of only part of the template (Figure S11).

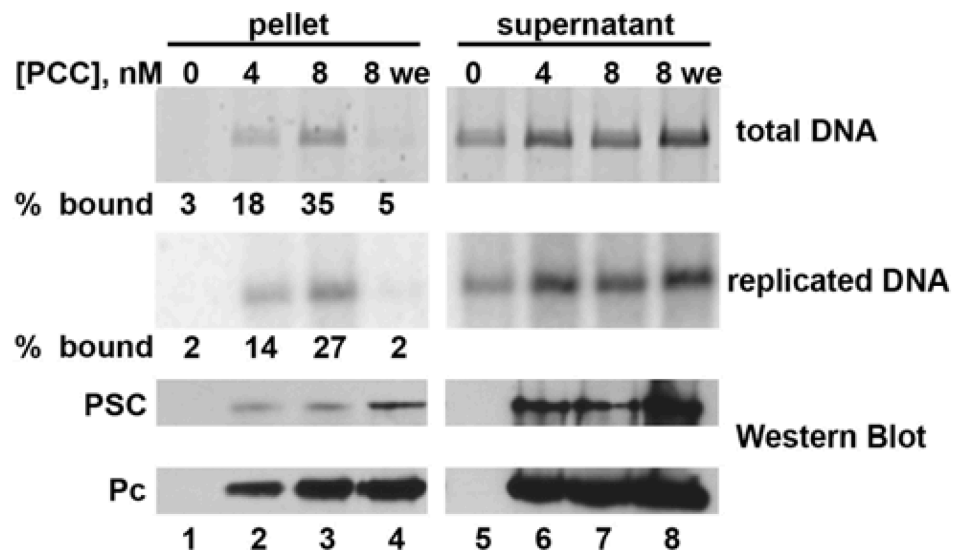
When naked DNA was used as the template for replication reactions, PCC could also be crosslinked to replicated and unreplicated templates (Figures 4C and 4D). A lower fraction of replicated than total DNA was captured when naked DNA was used as the template (compare Figures 4A and 4C), suggesting that nucleosomes help stabilize PCC on replicated or replicating templates. When PCC is added with replication extracts, near background levels of chromatin or DNA are retained on the beads, consistent with the conclusion that PCC does not bind chromatin or DNA under these conditions (Figures 4A and 4C lane 4). Bound and unbound replicated DNA was also digested with DpnI to confirm that PCC-bound





**Figure 4. PCC Can Be Crosslinked to Replicated Templates**

(A) Precipitation of replicated chromatin with biotinylated PCC after crosslinking. Top panels show total and replicated (radioactive) DNA, and the bottom panel shows a phosphorimager scan of the same reactions digested with DpnI. Lanes 1, 4, 5, and 8 contain 6% of the total reaction, lanes 2 and 6 18%, and lanes 3 and 7 54%. (B) Summary of fraction of replicated and total chromatin isolated with biotinylated PCC after replication of chromatin. Asterisks indicate that the fraction of replicated DNA precipitated is significantly lower than of total DNA ( $p < 0.05$ , two-tailed Student's  $t$  test). (C and D) Same as (A) and (B) except with naked DNA templates. Equal amounts of each reaction were loaded. Bottom panel is SYBR gold stain of DpnI-digested replication products. Lane 9 shows linearized and 10 completely DpnI digested template. In both (A) and (C), longer exposures are shown for the pellets than supernatants.



**Figure S11. Analysis of biotinylated protein capture in pull down experiments.**

Top two bands show total DNA (top) and replicated DNA in the beads or supernatant fractions from replication reactions with chromatin templates. Bottom two panels show western blotting for PSC and Pc for the same reactions. When template is added with the extracts (lanes 4 and 8), little template but substantial protein is recovered in the pellet fraction. These data also show that only a fraction of the protein is captured in all reactions, which likely explains why only a fraction of the DNA is captured.

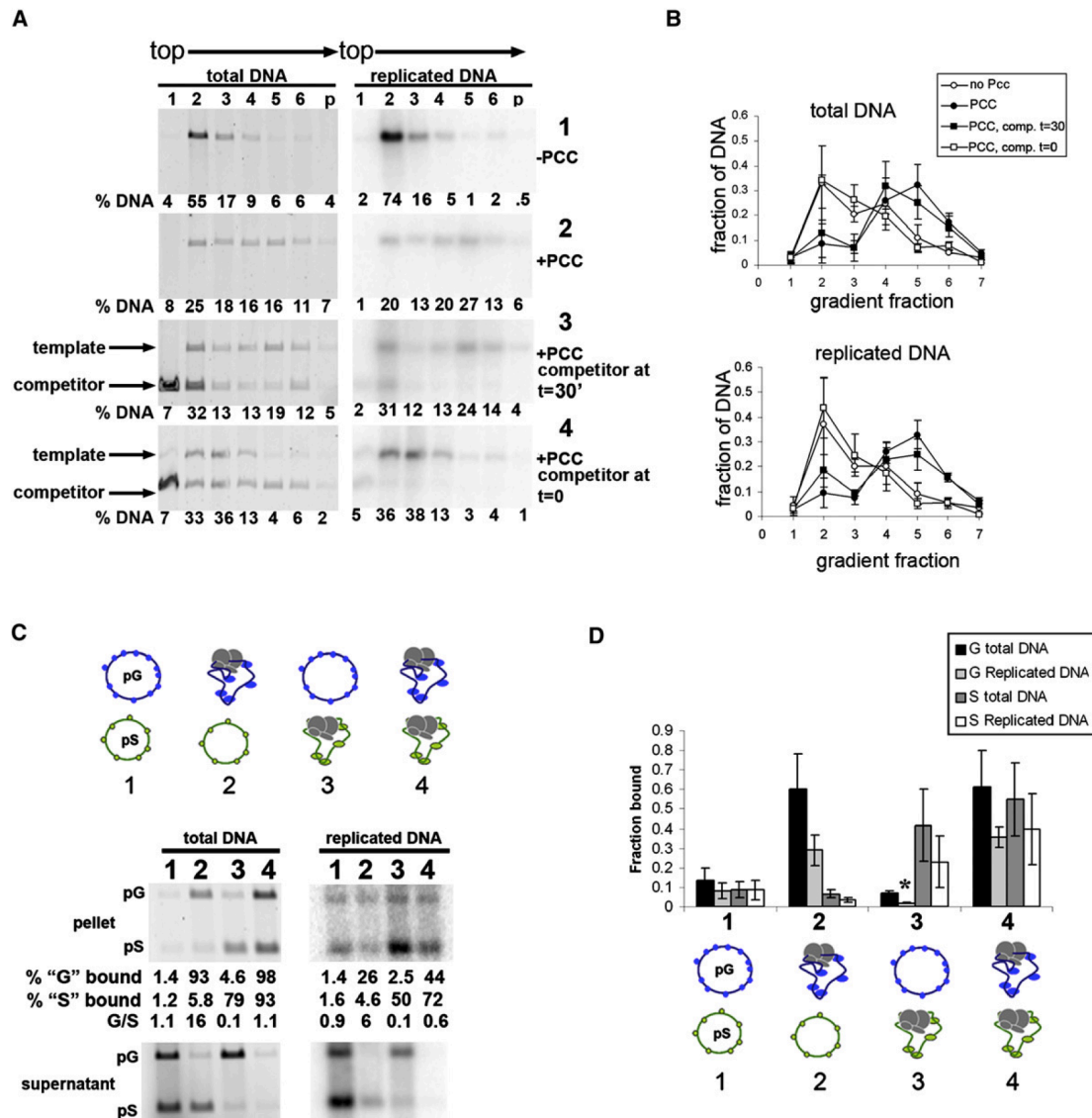
chromatin and DNA can be fully replicated (Figures 4A and 4C, bottom panels). We conclude that when PCC is bound to chromatin or naked DNA before DNA replication, it is bound after DNA replication.

#### *PCC Does Not Transfer among Templates during DNA Replication*

PCC is bound to replication products only if it is bound to the template prior to addition of replication extracts. The simplest explanation for this is that PCC is transferred to newly replicated chromatin without being released into solution, since if it was released even transiently, our data predict that it would not be able to rebind. We carried out two types of experiments to address whether PCC can transfer among DNA molecules during DNA replication.

We used naked DNA without a replication origin as a competitor to capture any PCC that was released during DNA replication. Competitor was added to replication reactions either with the replication extract or in the initial template binding step. Replication products were analyzed by sucrose gradient sedimentation (Figures 5A and 5B). When PCC was prebound to the template, competitor added during DNA replication had no effect on the association of replicated templates with PCC (panel 3 of Figure 5A). However, when the same amount of competitor was added during the binding reaction, PCC bound to the competitor and not detectably to the replicating template (panel 4).

To assess whether PCC can transfer among replicating templates, we used two plasmids that differ in size but both contain replication origins. Replication



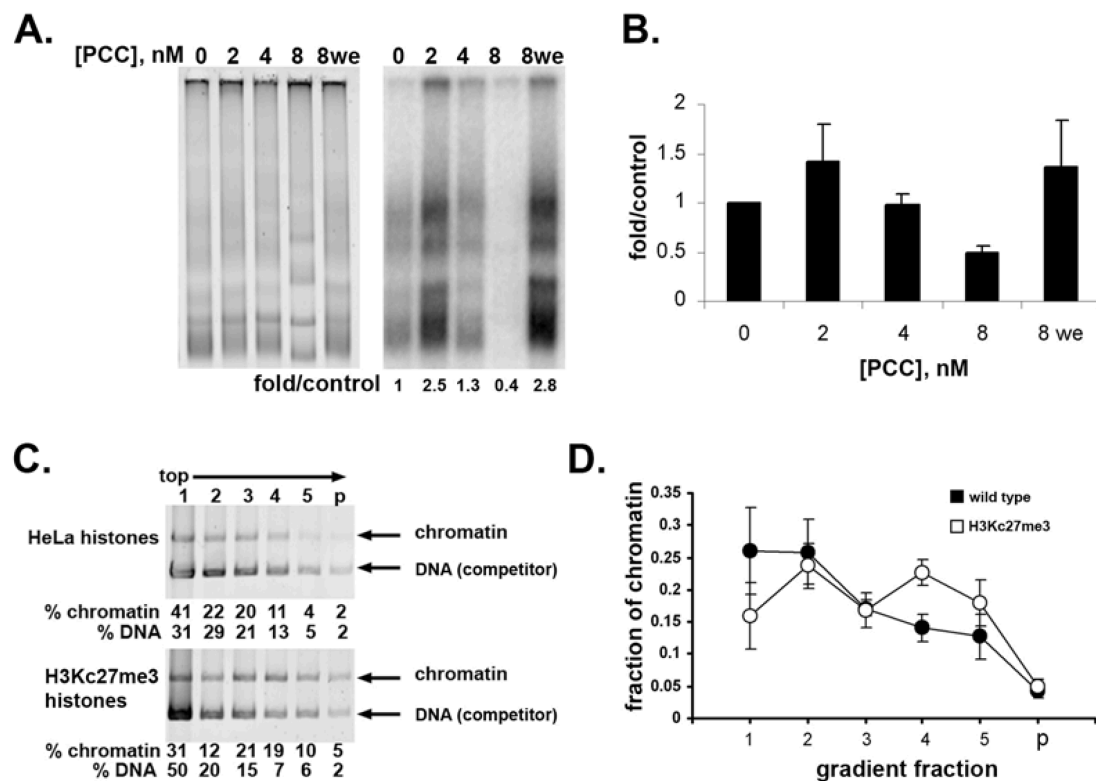
**Figure 5. PCC Does Not Transfer to Other DNA Templates during Replication**  
**(A)** Sucrose gradient sedimentation of replication reactions without competitor (panels 1 and 2), or with competitor added before ( $t = 0$ , panel 4) or after ( $t = 30$  min, panel 3) PCC binding. **(B)** Summary of sucrose gradient sedimentation experiments with competitor. **(C)** Precipitation of chromatin after replication of mixtures of PCC-bound and unbound plasmids. In each reaction, one, both or either plasmid was prebound with PCC as indicated. The plasmids were mixed when the replication extracts were added. The replicated DNA pellet samples were exposed for longer than the supernatant. For reaction 2, the amounts of replicated pG and pS in the bound fraction appear to be similar, but the fraction of the replicated templates that is bound indicates preferential binding of pG. **(D)** Summary of mixed plasmid experiments. Asterisks indicate cases where the fraction of replicated DNA that is precipitated with biotinylated PCC is significantly lower than the fraction of total DNA ( $p < 0.05$ , two-tailed student's  $t$  test).

reactions in which both, one, or neither plasmid were preincubated with biotinylated PCC were carried out. We found that the plasmid that was preincubated with biotinylated PCC prior to replication is preferentially retained on the streptavidin coated beads (Figures 5C and 5D). This was true both for replicated and unreplicated chromatin, even though more replication is observed from the plasmid that was not preincubated with PCC. These data suggest that PCC does not transfer among templates during replication *in vitro*.

#### *H3Kc27me3 Chromatin Allows Replication and Transfer of PCC*

*In vivo*, nucleosomes at or near PREs usually contain H3K27me3 (Papp and Müller, 2006; Schwartz et al., 2006). Our results indicate that once PCC is bound to chromatin (in this case, binding is driven by mass action), H3K27me3 is not required to maintain PCC through DNA replication. However, given that the Pc-H3K27me3 interaction is likely to occur at or near PREs *in vivo*, we wondered whether H3K27me3 would affect the replication of PCC-bound chromatin or the association of PCC with chromatin through DNA replication.

Histone octamers including histone H3 with a methyl-lysine analog (MLA) (Simon et al., 2007) at residue 27 that mimics H3K27me3 (H3Kc27me3) were assembled onto replication templates. PCC inhibits replication of H3Kc27me3 chromatin at similar concentrations as chromatin assembled with histones purified from cells (Figures S12A and S12B) or produced in bacteria (data not shown). A slight binding preference of PCC for H3Kc27me3 chromatin over control chromatin was



**Figure S12. Characterization of PCC interaction with H3Kc27me3 chromatin.**

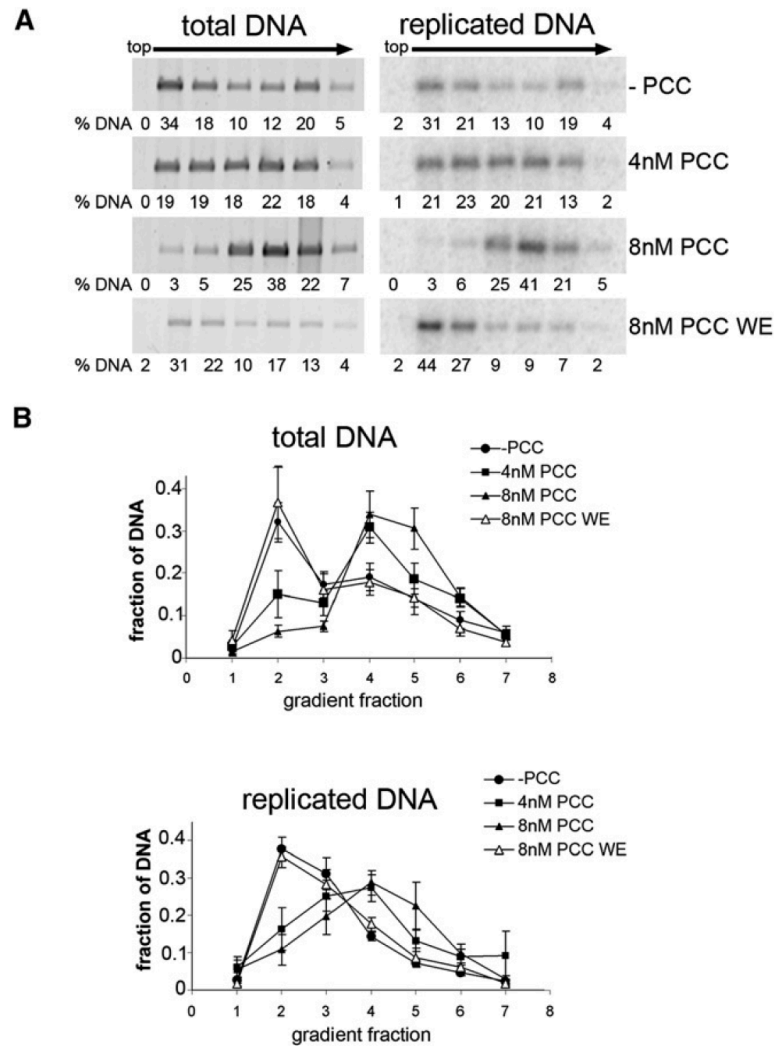
**A.** Replication of H3Kc27me3 chromatin in the presence of PCC. Left panel shows total DNA by SYBR gold staining, and right panel is a phosphorimager scan of replicated chromatin. **B.** Summary of inhibition of replication by PCC on H3Kc27me3 chromatin. All of these experiments were carried out with NPE-1000, although similar results were obtained in a smaller number of experiments without NPE-1000. **C.** Example of competition binding assay comparing chromatin templates assembled with HeLa histones or H3K27me3 MLA histones. Chromatin templates were mixed with a five-fold excess of naked DNA competitor (smaller plasmid) and incubated 30 minutes on ice to allow binding. Reactions were fractionated on sucrose gradients and DNA was isolated from each fraction and linearized so that the two plasmids could easily be distinguished. When H3Kc27me3 chromatin is used, more of the chromatin migrates lower in the gradient than when HeLa histone chromatin is used. **D.** Summary of multiple binding assays. Comparison of fractions 4 and 5 between the two data sets indicates a small increase in binding when H3K27me3 chromatin is used.

observed in the context of an excess of naked DNA competitor (Figures S12C and S12D); no difference in binding was detected in the absence of competitor. This is likely because both PCC and nucleosomal templates are many fold above their  $K_D$  in these experiments. Thus, it remains possible that there is a larger effect of H3K27me3 on chromatin affinity for PCC but that different conditions will be required to detect it. The MLAs may also bind less tightly to Pc than H3K27me3.

To determine whether PCC remains associated with replicated chromatin that includes H3K27me3 through DNA replication, we used sucrose gradient sedimentation to separate bound and unbound templates after replication (Figure 6). PCC remains associated with H3K27me3 templates through DNA replication. Thus, although H3K27me3 is not required for maintenance of PCC complexes during DNA replication *in vitro*, this more physiological substrate permits both DNA replication and maintenance of PCC.

#### *PcG Proteins Are Bound to Chromatin during S Phase and to Newly Replicated DNA In vivo*

Our data predict that PcG proteins are present on chromatin throughout DNA replication. Although the methods to directly test this *in vivo* are not yet available, we wondered whether we could detect PcG proteins on newly replicated chromatin. We synchronized *Drosophila* S2 cells at G1/S using a double thymidine block (Jackman and O'Connor, 2003) and then released them into media containing Bromodeoxyuridine (BrdU). Aliquots of cells were collected every 10 min as the cells



**Figure 6. Association of PCC with Chromatin that Has H3Kc27me3 through DNA Replication In Vitro**

(A) Sucrose gradient sedimentation of replication reactions carried out with H3Kc27me3 chromatin. These results are directly comparable to Figure S9. (B) Summary of multiple experiments with H3Kc27me3 chromatin. The H3Kc27me3 templates show a tendency to aggregate so that a fraction of the template migrates near the bottom of the gradient in the absence of PCC.



proceeded through S phase and were used to analyze BrdU incorporation, PcG protein binding, and colocalization of PSC and BrdU at three PREs in the Bithorax complex (BX-C) homeotic gene cluster (Figure 7C). We found that levels of PSC and Pc at the three PREs are fairly constant in cells undergoing S phase (Figures 7D and 7E). In control ChIPs without antibody, little PRE DNA was recovered, and little DNA corresponding to a previously characterized PcG-negative heterochromatin region was recovered with antibodies to PSC or Pc (Figures 7D and 7E) (Papp and Müller, 2006).

BrdU incorporation indicates that the BX-C is replicated within 90 min of release from the thymidine block, with the bulk of BrdU incorporation occurring between 20 and 60 min (Figures 7F–7H). To monitor PcG protein binding to replicated DNA, we carried out chromatin immunoprecipitation (ChIP) for PSC followed by immunoprecipitation with antibodies to BrdU (BrdU-IP). The enrichment of PSC-BrdU exactly follows the pattern of BrdU incorporation at the three PREs over the time course of replication (Figures 7F–7H, left panels). The specificity of the BrdU-IP is indicated by the low level of DNA recovered from chromatin harvested before addition of BrdU (“pre-BrdU”) or at the start of the time course (time = 0). We conclude that PSC is bound to DNA during or shortly after replication of DNA, although the time scale of these experiments would not detect rapid dissociation and rebinding.

We monitored PSC association with BrdU-labeled DNA out to 4 hr after release from the block. Interestingly, the level of PSC associated with BrdU-labeled

**Figure 7. Association of PcG Proteins from PRC1 with Polycomb Response Elements during DNA Replication**

(A) Strategy for analysis of PcG protein binding during DNA replication in S2 cells. (B) Representative FACS analysis of S2 cells arrested at the G1/S boundary using a double thymidine block. The final panel shows cells after 6 hr of the final thymidine treatment and is representative of the cell population at the start of the time course (8 hr). (C) Schematic diagram of part of the BX-C showing the position of three PREs used for this study, bx, bxd, and Mcp, and the position of the three homeotic genes in the complex (adapted from Figure 4 of Kwong et al., 2008). (D and E) ChIP for PSC (D) and Pc (E) at PREs and a negative control site throughout the time course of DNA replication. (F–H) Time course of BrdU incorporation (% input) and PSC-BrdU co-occupancy at three PREs over the time course of DNA replication. Data from sequential anti-PSC–anti-BrdU ChIPs are expressed as the percent of PSC-ChIP (since the PSC-ChIP elution is the input material for the BrdU IP in the sequential ChIP paradigm). Left panels show the first part of the time course, when replication is occurring (as demonstrated by BrdU incorporation). Right panels show the full 4 hr time course. Replication is largely completed by 90 min after release from the thymidine block for the sites analyzed; note that the broad peak of replication likely reflects imprecise synchronization of the cells, with some cells arrested in early S phase and others in G1. Bars in all graphs show the average of three experiments, and error bars show the standard deviation.

**A**

arrest *Drosophila* S2 cells at G1/S  
with double thymidine block

release cells into media with BrdU

collect cells every 10 minutes for ChIP

ChIP--PcG proteins    ChIP--BrdU    ChIP--PcG protein  
  ↓  
                                       ChIP--BrdU

**B**

starting population      10 hours in thymidine  
(block 1)

number of cells vs DNA content (propidium iodide stain)

22% G1  
49% S  
29% G2

10% G1  
72% S  
17% G2

release, 6 hours      6 hours in thymidine  
(block 2)

number of cells vs DNA content (propidium iodide stain)

19% G1  
67% S  
14% G2

88% G1  
10% S  
2% G2

**C**

12,500,00    12,550,00    12,600,00    12,650,00    12,700,00    12,750,00    12,800,00

Ubx    abd-A    Abd-B

bx    bxd    Mcp

**D**

PSC ChIP

% input

Time, minutes

■ bx  
▒ bxd  
□ Mcp  
○ non-PRE

**E**

Pc ChIP

% input

Time, minutes

■ bx  
▒ bxd  
□ Mcp  
○ non-PRE

**F**

bx

% input

Time, minutes

■ PSC-BrdU  
□ BrdU

**G**

bxd    bcd

% input

Time, minutes

■ PSC-BrdU  
□ BrdU

**H**

Mcp

% input

Time, minutes

■ PSC-BrdU  
□ BrdU

PRE DNA continues to increase after replication of these sequences is complete (Figures 7F–7H, right panels). These data are consistent with a model in which PSC bound before DNA replication is maintained on the daughter templates, and additional PSC is added after replication to restore the full complement required for silencing, although higher-resolution methods will be needed to confirm this.

## Discussion

We examined the effect of DNA replication on the association of PRC1-class complexes with chromatin and DNA using a cell-free system. Our principle finding is that PRC1-class complexes bound to chromatin or DNA remain associated during DNA replication *in vitro*. These results suggest that transfer of chromatin regulatory proteins may be a mechanism for epigenetic inheritance through cell division.

### *How Is PCC Retained on Replicating Templates?*

Our data suggest that PCC is not released into solution during passage of the DNA replication fork. Furthermore, nucleosomes facilitate PCC binding to and retention on templates, but are not essential for either. The finding that PCC can be maintained on either chromatin or naked DNA is interesting in light of the finding that PREs are sites of rapid histone turnover and can be depleted of nucleosomes (Mishra et al., 2001; Mito et al., 2007; Papp and Müller, 2006).

One model for the transfer of PCC during DNA replication is that the complex remains in direct contact with DNA during passage of the DNA replication fork.

Contacts between PcG proteins and nucleosomes or DNA could be disrupted in front of the replication fork, but replaced by contacts with nucleosomes or DNA behind the replication fork. This mechanism has been proposed for transfers of histone-DNA contacts during replication and transcription *in vitro* (Bonne-Andrea et al., 1990; Clark and Felsenfeld, 1992; Studitsky et al., 1994). PCC can likely contact multiple nucleosomes or a long stretch of DNA (Francis et al., 2004; Mohd-Sarip et al., 2006), which may allow the complex to remain on chromatin when some template contacts are disrupted. A second model is that PCC interacts with the replication machinery, either directly or through intermediary factors. These interactions could retain PCC near DNA during replication, even if direct DNA contacts are disrupted, allowing rapid rebinding of PCC to newly replicated chromatin. Consistent with this idea, several chromatin-modifying proteins can interact with components of the DNA replication machinery (Groth et al., 2007; Kohn et al., 2008).

#### *PCC Inhibits DNA Replication In vitro*

The inhibition of DNA and chromatin replication by PCC *in vitro* raises the question of how PcG-bound regions are replicated if PRC1-class complexes are indeed continuously bound. If PCC inhibits replication initiation but not elongation, as our results suggest, then PRC1-class complexes would limit replication only if they were bound near replication origins.

Intriguingly, targeting of Pc to a replication origin in *Drosophila* that mediates

developmental chorion gene amplification in follicle cells decreased gene amplification (Aggarwal and Calvi, 2004) and PcG-silenced regions of polytene chromosomes (such as Hox gene clusters) are underreplicated, although this involves additional genes such as Suppressor of DNA Underreplication (Marchetti et al., 2003; Moshkin et al., 2001).

*How Might Retention of PCC through DNA Replication Contribute to Heritable Transcriptional Silencing?*

Reduction of PcG protein levels leads to reactivation of their target genes, suggesting that these genes are continuously susceptible to transcriptional activation (see for example (Beuchle et al., 2001; Breiling et al., 2001; Cao et al., 2002; Wang et al., 2004b). It may therefore be important that PRC1-class complexes, which can directly repress transcription (King et al., 2002), maintain constant association with genes marked for silencing.

We were surprised to find that H3K27me3 is not essential for maintaining PRC1-class complexes through DNA replication *in vitro*. It is possible that retention of parental PRC1-class complexes and recruitment of new complexes are mechanistically distinct because we do not find evidence for recruitment of new PCC during replication, and our *in vivo* data suggest that PSC is present on newly replicated chromatin but that additional PSC is recruited after replication. This may be similar to histone proteins in that it is thought that parental histones are transferred randomly to the two daughter strands, followed by deposition of new

histones by replication-coupled assembly complexes (reviewed in (Groth et al., 2007)). Our *in vivo* data raise the possibility that recruitment of new PRC1 is not directly coupled to DNA replication; perhaps it involves H3K27me3.

In our experiments, PCC interacts with chromatin through mass action, but *in vivo*, PRC1-class complexes are specifically targeted to PREs. We hypothesize that the stable association of PCC with chromatin that we observe here reflects how the complex could behave once it is recruited to a PRE, but it will be important to test this mechanism in a system where PCC is targeted.

In conclusion, the ability of parental PCC to be transferred to daughter chromatin may help explain how PcG-mediated repression established by transiently acting factors can be propagated through cell generations. Our data also suggest that maintenance of chromatin regulatory proteins through DNA replication might be an important mechanism of epigenetic inheritance.

## **Experimental Procedures**

### *Chromatin Templates*

The HindIII-AvrII fragment of pSVO11+ (Stillman and Gluzman, 1985) was cloned into pG5E4 (Utlei et al., 1998) to produce pG5E4-SVO, which includes the SV40 origin flanked by five 5S nucleosome positioning sequences on each side. Plasmids were assembled into chromatin by salt gradient dialysis with histones purified from HeLa cells or prepared with H3Kc27me3 MLAs (Carruthers et al., 1999; Schnitzler, 2001; Sif et al., 2001; Simon et al., 2007) in the presence of

Topoisomerase I (Figure S1).

### *Proteins*

PCC was prepared as described; all stated concentrations refer to active concentration (Francis et al., 2001; Lavigne et al., 2004). TAg was prepared by immunoaffinity chromatography (Lanford, 1988). Labeling of PCC with maliemide-biotin, Cy5, or Cy3 was carried out in BC300N (20 mM HEPES, 0.2 mM EDTA, 300 mM KCl, 20% Glycerol, 0.05% NP40) (pH 7.2) for 5 min at room temperature. Reactions were stopped with  $\beta$ -mercaptoethanol; free label was removed by spin-column chromatography and proteins dialyzed back into BC300N (pH 7.9). S100 extracts for replication were prepared from HeLa cells (Abmayr et al., 2006).

### *Xenopus laevis Nucleoplasmic Extract Preparation and Fractionation*

Nucleoplasmic extract (NPE) (Walter et al., 1998) was bound to a Q-sepharose column in 150 mM NaCl and eluted at 350 and 1000 mM NaCl. The 1000 mM NaCl eluate (NPE-1000) was desalted, concentrated, and used to stimulate replication.

### *Replication Reactions*

Replication was carried out essentially as described (Stillman and Gluzman, 1985) except that TAg was prebound for 20 min at 37°C, followed by binding of PCC for 15 min at 30°C. Replication extract with NTPs and dNTPs was then added for 2 hr



at 30°C. Replication reactions were stopped with 1% SDS and 0.1 M EDTA, digested with Proteinase K and RNase A, and either analyzed on agarose gels or phenol-chloroform extracted, ethanol precipitated, and digested with restriction enzymes.

#### *Restriction Enzyme Accessibility Assay for Inhibition of Chromatin Remodeling*

After DNA replication, 1 µl of the replication reaction was used in a 40 µl remodeling reaction as described (Francis et al., 2001).

#### *Sucrose Gradient Sedimentation*

Sucrose gradients were prepared by layering sucrose solutions of 10% increments made in BC buffer with 150 mM KCl and sucrose in place of glycerol from 80%–30% for chromatin or 70%–30% for naked DNA. For minigradients, layers were 80%, 40%, 20% for chromatin and 80%, 60%, 40%, and 20% for naked DNA. Gradients were centrifuged in a TLS-55 rotor in an Optima table top ultra centrifuge (Beckman Coulter) at 55,000 rpm for 60–90 min for large gradients, and 40,000 rpm for 45–75 min for small gradients.

#### *Crosslinking and Precipitation with Biotinylated PCC*

Completed replication reactions were crosslinked with formaldehyde. Chromatin was pelleted by centrifugation, washed, resuspended, and bound overnight to streptavidin-coated Dynabeads (M-280, Invitrogen). Crosslinks were reversed, and DNA was purified, digested with restriction enzymes, and analyzed on

agarose gels.

### *Synchronization of Drosophila S2 Cells and Chromatin Immunoprecipitation*

*Drosophila* S2 cells (obtained from the American Type Culture Collection) were synchronized by double block with 2 mM Thymidine (Sigma) according to the following scheme: 10 hr block—6 hr release—8 hr block. After the second block, cells were transferred to thymidine free, BrdU-containing media. At each time point, cells were fixed and frozen; cell pellets were processed for ChIP according to standard protocols (based on protocols from Upstate Biotechnology) (Kind et al., 2008; Rastegar et al., 2004; Schwartz et al., 2005). Anti-Pc antibody for ChIP was a gift from J. Müller (European Molecular Biology Laboratory, Heidelberg) (Papp and Müller, 2006); anti-PSC was prepared for this study. Purified DNA was analyzed by real-time PCR.

Anti-BrdU immunoprecipitations (BrdU-IP) were carried out according to (Azuara, 2006). For single BrdU-IP, crosslinks were reversed, and DNA was purified and used for BrdU-IP. For PSC-BrdU double ChIP experiments, ChIP eluates were used after crosslink reversal and DNA purification.

## **Supplemental Experimental Procedures**

**Reagents and enzymes:** Restriction enzymes and Topoisomerase I were purchased from New England Biolabs (Ipswich, MA), Topoisomerase II from TopoGen (Port

Orange, FL), Micrococcal nuclease from USB (Cleveland, OH), RNase A from Sigma (St. Louis, MO), Proteinase K from VWR (Westchester, PA), SYBR gold from Invitrogen (Carlsbad, CA) and  $\alpha$ 32P-dATP from Perkin Elmer (Waltham, MA) . Maleimide-PEO-2-biotin was purchased from Pierce Biotechnology (Rockford, IL), and Cy3 and Cy5 Maleimide mono-reactive dyes from GE Healthcare (Piscataway, NJ).

**Chromatin templates:** The HindIII-AvrII fragment of pSVO11+ (obtained from B. Stillman; (Stillman and Gluzman, 1985) was cloned into pG5E4 (described in (Utley et al., 1998), to produce pG5E4-SVO which has the SV40 origin in place of the promoter and Gal 4 binding sites in the original pG5E4 (sFig. 1A). Plasmids were assembled into chromatin by salt gradient dialysis as described (Carruthers et al., 1999; Sif et al., 2001) except that Topoisomerase I (5U) was included (sFig. 1).

**Proteins:** PRC1 core complex (PCC) preparation and measurement of active molecules by DNA binding were as described (Francis et al., 2001; Lavigne et al., 2004). All stated concentrations refer to active concentration. SV40 large T Antigen (TAg) was prepared by immunoaffinity chromatography as described (Landford, 1988) using pAB419 (gift from B. Stillman). To label PCC with biotin, Cy5 or Cy3, protein was dialyzed into labeling buffer (20 mM Hepes, pH 7.2, 0.4 mM EDTA, 300 mM KCl, 20% glycerol, 0.05% NP40, 0.2 mM PMSF) and treated 30 minutes at room temperature with a 100-fold excess of TCEP. Maleimide-PEO-2-biotin resuspended in

labeling buffer was added at 1:10 or Cy5 or Cy3 monoreactive dyes in DMSO at 1:20, and reactions were incubated 5 min. at room temperature.  $\beta$ -mercaptoethanol was added to 80 nM and reactions were centrifuged through two G50 micro-spin columns (GE Healthcare). Cysteine was added to 20 mM and the protein dialyzed (2\*0.5 L BC300N with 10mM cysteine; 2\*0.5 L BC300N; BC300N: 20 mM Hepes, pH 7.9, 0.4 mM EDTA, 300 mM KCl, 20% glycerol, 0.05% NP40, 0.2 mM PMSF, 0.5 mM DTT). DNA binding activity of labeled PCC was tested by filter binding; biotinylation or labeling with fluorophores did not significantly decrease the active fraction of PCC.

Histones were purified from spent HeLa cell nuclear pellets using hydroxyapatite chromatography (Schnitzler, 2001). S100 extracts used as the source of replication factors were prepared (Abmayr et al., 2006) from HeLa cells (HeLa-Ini1 cells, National Cell Culture Center, Minneapolis, MN). H3Kc27me3 MLAs were prepared as described (Simon et al., 2007) and were assembled into histone octamers with bacterially produced *Xenopus* histones. Octamers were purified as described (Luger et al., 1999).

***Xenopus laevis* nucleoplasmic extract preparation and fractionation: *Xenopus***

Nucleoplasmic extract (NPE) was prepared from *Xenopus* eggs as described (Walter et al., 1998). 350-500  $\mu$ l of NPE (which is in 1X ELB: 10 mM Hepes, pH 7.7, 2.5 mM MgCl<sub>2</sub>, 50 mM KCl, 250 mM sucrose) was adjusted to 150 mM NaCl, 4 mM MgCl<sub>2</sub>, 1X Energy mix (2 mM ATP, 5  $\mu$ g/ $\mu$ l Creatine Kinase, 20 mM Creatine Phosphate) and

aprotinin/leupeptin (10 µg/ml) and loaded onto a 1 ml Q-sepharose FF column equilibrated with ELB adjusted to 150 mM NaCl, 4 mM MgCl<sub>2</sub> and 2 mM ATP using an AKTA FPLC. Bound proteins were eluted in two steps of 350 and 1000 mM NaCl. Peak fractions from the 1000 mM NaCl elution were concentrated to 0.5-1.5 mg/ml, desalted into (ELB+ energy mix) using a Centriprep 10 KDa cutoff concentrator (Millipore, Billerica, MA), and stored at -80°C as NPE-1000. At least 5 preparations were used in these experiments. NPE, flow through fractions, and the 350 mM elution did not stimulate replication in the SV40 system.

**Replication reactions:** 25ng of template were incubated with saturating amounts of TAg in 10 mM Hepes, 0.1 mM EDTA, 10% glycerol, 2.5 mM ATP, 5 mM MgCl<sub>2</sub>, 0.25 U of Topoisomerase I, 1.25 mM DTT, for 20 minutes at 37°C. 1 µl of PCC complex or BC300N buffer were added for 15' at 30°C. The concentration of nucleosomes at this step is 40 nM, and of plasmids is 1.3 nM. Replication mix was added, which contained 5 µl of S100 extract, 0.6 µl of replication cocktail (8 mM DTT, 8 mM MgCl<sub>2</sub>, 7X energy mix, 0.28 mM dATP, 1.4 mM dCTP, 1.4 mM dGTP, 1.4 mM dTTP, 2.8 mM each NTP), 0.05 µl of Topoisomerase I, 0.2-0.4 µl of α-<sup>32</sup>P-dATP, and 2-3 µl of NPE-1000 fraction for 2 hours at 30°C. In some experiments, 3 U Topoisomerase II (Topo II) (3U) was added with the replication mix, along with 1µl of Topo II reaction buffer, ATP to 6mM, and MgCl<sub>2</sub> to 10mM, and reactions were incubated 15 min. at 37 °C at the end of the replication reaction. Note that the effect of PCC on replication was also observed if TAg was not prebound but just added with the

replication extracts. Replication reactions were stopped with 3 µl of DSB-PK (50 mM Tris, pH 8, 0.1 M EDTA, 1% SDS, 25% glycerol, xylene cyanol, bromophenol blue and 2 µl of 20 mg/ml Proteinase K) with 0.8 mg/ml RNase A, digested overnight, and either loaded directly onto native agarose gels or extracted with phenolchloroform and chloroform and precipitated with ethanol, sodium acetate, and tRNA for further analysis.

**Analysis of replication products:** Conditions for DpnI digestion were (Sanchez et al., 1992): 50 mM KCl, 5 mM MgCl<sub>2</sub>, 20 mM Tris, pH 7.4, 0.1 µg/ml BSA, 3mM Spermidine, 0.01% Triton X-100, 1 U DpnI, 4 U NdeI, RNase A (2 µg), and water to 20 µl (Sanchez et al., 1992. In some cases, 300ng of lambda DNA was added as a carrier (Sanchez et al., 1992). Reactions were incubated 30-60 min. at 37°C. Digestion with NdeI or NdeI, AlwNI, and ClaI was carried out with 4 U of each restriction enzyme for at least 2 hours. Reactions were separated on 1X TAE agarose gels, stained with SYBR gold, scanned on a Typhoon phosphorimager, and transferred to Hybond+ membranes (GE Healthcare). Membranes were exposed to phosphorimager screens for quantification using ImageQuant. To calculate the ratio of actual to expected incorporation for reactions digested with NdeI, AlwNI, and ClaI (Fig. 2B, D), actual incorporation was determined by quantifying the radioactivity in each band and expressing it as a fraction of the total signal from all three bands; expected incorporation was calculated as the fraction of total A residues in the plasmid that are present in each fragment.

For denaturing gels, reactions were diluted 1:10 in alkaline loading buffer (60 mM NaOH, 2 mM EDTA, pH 8.0, 20% Ficoll). Alkaline agarose gels were prepared by melting agarose in water, and adding NaOH to 30 mM and EDTA to 1 mM just before pouring. Gels were allowed to equilibrate with denaturing buffer (30 mM NaOH, 1 mM EDTA), and pre-run at 50 V for 30 minutes prior to loading. Gels were run at 100 V for 2-3 hours, at 4 °C and with one or two buffer changes as necessary.

**Restriction enzyme accessibility assay for inhibition of chromatin remodeling:**

Assays were carried out as described (Francis et al., 2001) with 1 µl of each replication reaction (~5 ng of chromatin) in a 40 µl remodeling reaction. % inhibition was calculated using the following equation:

$$\% \text{ inhibition} = \{(\% \text{ uncut with hSwi/Snf and PCC} - \% \text{ uncut with hSwi/Snf}) / (\% \text{ uncut without hSwi/Snf} - \% \text{ uncut with hSwi/Snf})\} * 100.$$

**Sucrose gradient sedimentation:** Sucrose solutions contained 20mM Hepes, 0.4 mM EDTA, 150 mM KCl, 0.05% NP40, 0.5 mM DTT, 0.2mM PMSF and gradients were spun in a TLS-55 rotor in an Optima table top ultra centrifuge (Beckman Coulter) at 55,000 rpm for 60-90 min. for large gradients and 40,000 rpm for 45-75 min. for small gradients. (Beckman Coulter). Fractions were removed from the top of gradients by pipetting. To prepare large gradients for separation of chromatin and DNA, sucrose solutions were sequentially layered: chromatin: 250 µl of 80%, 375 µl of 70%, 60%, 50%, 40% and 30%; naked DNA: 250 µl of 70%, 470 µl of 60%, 50%,

40% and 30%. 30  $\mu$ l of the sample was mixed with 30  $\mu$ l of the top layer, which was then layered on top of the gradient; the remainder of the sample was layered on top of this. Mini sucrose gradients from separation of replication reactions carried out with chromatin contained 80  $\mu$ l of 80%, 120  $\mu$ l of 40% and 40  $\mu$ l of 20% sucrose; for naked DNA, 80  $\mu$ l each of 80%, 60%, 40% and 20% sucrose.

**Cross-linking and precipitation with biotinylated PCC:** For chromatin precipitation reactions, replication reactions were carried out with biotinylated PCC. Replication reactions were cross-linked 5 min. with 1% formaldehyde and quenched 5 min. with 125 mM glycine at room temperature, diluted 4 fold with BC100 (20 mM Hepes, 0.4 mM EDTA, 100 mM KCl), and loaded onto 150  $\mu$ l of BC100 with 20% glycerol. They were centrifuged for 30 min. at 55,000 rpm in a table top ultracentrifuge; supernatants were discarded and pellets washed once with 150  $\mu$ l of BC100 and centrifuged 15 min. at 55,000 rpm. Pellets were resuspended in 100  $\mu$ l of BC300N with 2 mM MgCl<sub>2</sub> and 10-12  $\mu$ l of streptavidin coated Dynabeads were added (M-280, Invitrogen). Dynabeads were prepared by washing with TB (10 mM Tris, pH 7.7, 1 mM EDTA, 1 M NaCl) and three times in binding buffer. After binding overnight at 4°C, beads were captured, and washed 2X with 100  $\mu$ l wash buffer 1 (50 mM Tris, pH 8, 2 mM EDTA), 1X with wash buffer 2 (10 mM Tris, pH 8, 1 mM EDTA, 2% SDS, and 1X with wash buffer 1 at room temperature. Beads were resuspended in elution buffer (10 mM Tris, pH 8, 1 mM EDTA, 200 mM NaCl, 1% SDS), Proteinase K was added to both beads and supernatant, and supernatant was brought up to 0.1% SDS



and 400 mM NaCl. Both beads and supernatant were incubated for 4 hours at 65°C to reverse the cross-links, and the DNA was purified and analyzed as above.

#### **Synchronization and Chromatin Immunoprecipitation from *Drosophila* S2 cells:**

*Drosophila* S2 cells (obtained from the ATCC) were grown in ESF 921 media (Expression Systems, Woodlawn, CA) at a density of between 1 and 6\*10<sup>6</sup> cells/ml in shaking flasks at 27°C. To synchronize cells, cultures at 2-2.5 \*10<sup>6</sup> cells/ml were treated for 10 hours with 2mM Thymidine (Sigma). To release cells from the block, cells were isolated by centrifugation (1200 rpm, 2 minutes), washed, and resuspended in fresh media; cell density was adjusted to ~2.5\*10<sup>6</sup> cells/ml. After 6 hours, Thymidine was added to the cultures again to 2mM. After 8 hours in the second thymidine block, cells were counted, washed, and resuspended in fresh media without thymidine but containing 50 µM BrdU (Sigma). An aliquot of cells was collected at each stage in the synchronization procedure and analyzed for DNA content by FACS using propidium iodide stain and an LSRII cell sorter (BD Biosciences) (Fig. 7B). At each time point, cells were fixed for 10 minutes with 1% formaldehyde, and glycine was added to 0.125 mM to quench the formaldehyde for 5 minutes. Cells were pelleted, washed twice with 1X PBS and cell pellets frozen in liquid nitrogen.

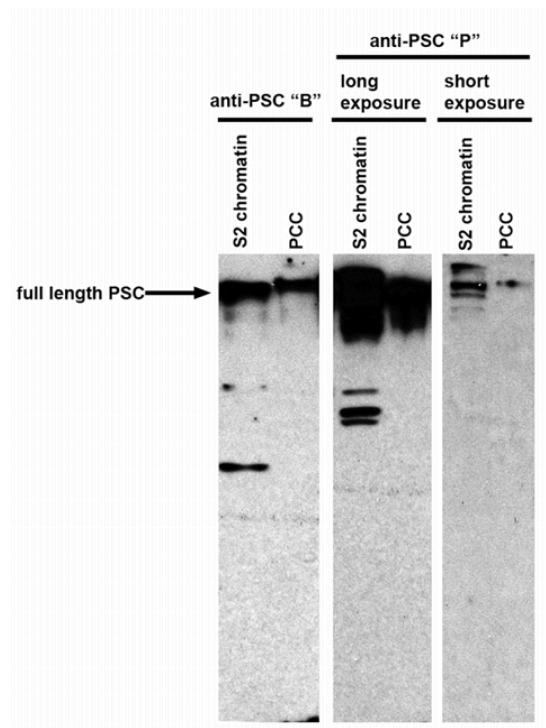
For ChIP experiments, chromatin was prepared as follows (based on protocols from Upstate Biotechnology; (Kind et al., 2008; Rastegar et al., 2004; Schwartz et al., 2005)). Pellets were thawed and resuspended in 5 ml of wash buffer

I (10 mM Hepes, pH 7.6, 10 mM EDTA, 0.5 mM EGTA, 0.25% Triton X-100), incubated 10 minutes at 4°C with rotation, centrifuged at 1200 rpm for 2 minutes, and resuspended in 5 ml of wash buffer II (10mM Hepes, pH 7.6, 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.01% Triton X-100), incubated 10 min. at 4°C with rotation and centrifuged at 1200 rpm for 2 minutes. Pellets were resuspended in sonication buffer (50 mM Hepes, pH 7.5, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS) to a cell concentration of  $2 \times 10^7$  cells/ml (4 or 5 ml for each time point), and sonicated with 12\*30 second pulses with 1 minute between pulses using a Sonics Vibracell sonicator at 40% power. Sonications were checked by agarose gel electrophoresis, and fragments were mainly .5-1kb. Following sonication, samples were adjusted to RIPA buffer (1% Triton X-100, 0.1% SDS, 0.1% sodium deoxycholate, 1 mM PMSF), and centrifuged for 5 minutes at full speed in a refrigerated microcentrifuge. The supernatant was used for ChIP. For single ChIP experiments, 400  $\mu$ l of chromatin (corresponding to  $\sim 8 \times 10^6$  cells) was used; this reaction was scaled up 5-fold for the double ChIP-BrdU IP experiments. Samples were precleared with protein A agarose beads (Sigma) blocked with BSA and salmon sperm DNA (ssDNA) (Invitrogen) (for 5 ml of packed beads: 5 ml of 10 mM Tris, pH 8.0, 1mM EDTA, 0.05% sodium azide; 2-4 mg ssDNA, 5 mg BSA ). Antibody was added and samples were incubated overnight at 4°C with rotation. The anti-Pc antibody was a gift from anti-Pc, J. Müller (EMBL, Heidelberg) (Papp and Muller, 2006). The anti PSC rabbit antisera were prepared for this study. Two antisera were used, one of which was raised against PSC aa 521-869 ("B") and

the other against PSC aa 819-926 ("P"). Antibodies were affinity purified and a mixture of the two antisera used for ChIP experiments (2 µg of "P" and 4 µg of "B" were used for 400 µl of chromatin). A Western blot demonstrating the specificity of these antibodies is shown in sFig. 13. The next day, beads were added to capture immune complexes for 1-2 hours at 4°C. Beads were washed once each of the following buffers: low salt (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris, pH 8.0, 150 mM NaCl), high salt (same as low salt but 500 mM NaCl), lithium chloride (0.25 M lithium chloride), 1% NP40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris, pH 8.0) followed by 2 washes in TE, pH 8.0. For each wash, beads were resuspended in 1 ml buffer per 30 µl of bead slurry, gently vortexed, and incubated 5 minutes at 4°C with rotation. To isolate beads, reactions were centrifuged 1000 rpm for 2 minutes. To elute DNA from beads, beads were incubated with elution buffer (1% SDS, 0.1 M sodium bicarbonate) twice for 15 minutes at room temperature. Elutions were pooled, adjusted to 200mM NaCl, and incubated at least 4 hours at 65°C to undo cross-links. Samples were treated for 1 hour with Proteinase K, extracted with phenol-chloroform-isoamyl alcohol, and ethanol precipitated with yeast tRNA and sodium acetate. After centrifugation and washing with 70% ethanol, air dried pellets were resuspended in 100 µl of ddH<sub>2</sub>O for PCR or in 500 µl of TE, pH 7.4 for BrdU immunoprecipitation. Real time PCR was carried out on a Bio-Rad IQ5 machine using SYBR green (Bio-Rad). 5 µl of template was used in a 20 µl reaction. Standards ranged from 100 to 0.1 ng of genomic DNA from S2 cells and 40 cycles of PCR were run. Detection was carried out at 76°C. R<sup>2</sup> values for the standard curves were all

above 0.9. Three points were discarded from the analysis because the amount of DNA detected by PCR was at least three fold less than that for the points on either side.

For anti-BrdU immunoprecipitations (BrdU-IP), we adapted a protocol for measuring replication timing from small numbers of cells (Azulara, 2006) (all buffer recipes are detailed in this reference). For single Brd-IP, 200  $\mu$ l of chromatin was treated to reverse cross-links as described above; DNA was purified and used for BrdU-IP. For PSC-BrdU double ChIP experiments, ChIP eluates were used after cross-link reversal and DNA purification. Salmon sperm DNA (20  $\mu$ l of 10mg/ml) was added to each sample, and samples were boiled 5 minutes and put on ice for 1 minutes. Adjustment buffer was added, followed by anti-BrdU (60-80  $\mu$ l, BD Biosciences, Franklin Lakes, NJ, 347580). Samples were incubated 20 minutes at room temperature with rotation. Goat anti-mouse IgG (35  $\mu$ g) (Sigma, M-7023) was added for 20 minutes at room temperature, and antibody complexes were harvested by centrifugation at full speed in a refrigerated microfuge for 15 minutes. Pellets were washed once with washing buffer, re-centrifuged, and resuspended in lysis buffer II. Samples were digested for 2 hours at 50°C, and DNA purified by phenol-chloroform extraction as above. Samples were dissolved in water and used for PCR as described above.



**Figure S13. Characterization of anti-PSC antibodies.**

Western blot of *Drosophila* S2 cell chromatin and recombinant PSC demonstrating that anti-PSC antibodies strongly recognize full-length PSC. For the "P" antiserum, the blot on the left is the same exposure as the "B" antiserum, while the one on the right is a much shorter exposure. Approximately 5 fm of PCC were loaded as a positive control.

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